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(54) Title: SCREENING TESTS FOR LYTIC CYCLE ANTIGENS AND ANTIBODIES TO KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS

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-120  attcgagacctacatcaaaactgtcagttttgtagtgcccttggccttgggacgccccgactgc
-60  gagggctgccctattaagcaccgtgacgtgcccggactgcttcagctcaccctcacagctg
1    CTGGAOTICTOTTCGCGQAAGTOTTCCTCCTGAGGCTATTTCCGCCCCCTGTGTGGAAGA
61  TGTCCAACITTAAGGTGAGAGACCCCGTGATCCAGGAGCGACTGGATCATGACTACGGCTC
    S N F K V R D P V I Q E R L D H D Y A H
121  ACCATCCCTTGTGGCTCCGATGAATACCCCTGGATCAGGGAAATATGTCGCGAGCCGAAT
    H P L V A R M N T L D Q G N M S Q A E Y
181  ACCTCGTGCAAAAGAGACACTATCTCGTOTTCTTAATTGCCCATATTATATGAGGCCCT
    L V Q X R H Y L V F L I A H H Y Y E A Y
241  ATCTGAGGAGGATGGGTGGAAATCAAAAGCCGGGATCATCTOCAGACCCTTAGGGATCAGA
    L R R M G G I Q R R D H L Q T L R D Q K
301  AACCOCGCGAGCCCGCTGACCGAGTTTCCCGGCGCTTACGACCGCGGGACGTTCA
    P R E R A D R V S A A S A Y D A G T F T
361  CCGTCCCTTCGAGCCAGGCCCTGCATCCCGCACCACCCCGGGGCGCAGGACTCACTGG
    P S R P G P A S G T T P G G Q D S L V G
421  GGGTCTCGGAAGCAOTATAACCAACCTGTCTCTGGTCCCGCATTCATTGTCCCGCCGT
    V S G S S I T T L S S O P H S L S P A S
481  CAGACATCTCACACCCCTCTCATCCACGACGGAAACCGCCCGCCCGCGGTGGCCGAGC
    D I L T T L S S T T E T A A P A V A D A
541  CGAGGAAACCCCTCTGGCAAAAGAAATAGGCAACGATTGGACAACCGTGGAGTCACA
    R K P P S G K K K *
601  AGTACCACCTTCTTTATCTGTCAACCGTCTCCGCCAGGGACCGGATAGGCGGCCACTT
661  TTTGTTGATGCGCTCTAATAAACTAATCAAGTGTATCAAAAAAAAAAAAAAAAAAAAA
  
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(57) Abstract

A screening test for Kaposi's sarcoma-associated herpesvirus in a patient involves obtaining a biological sample from the patient, and assaying for the presence of lytic cycle antigens or antibodies in the sample. In preferred embodiments, fluid samples such as peripheral blood mononuclear cells are screened for antigen of Kaposi's sarcoma-associated herpesvirus, e.g., small viral capsid antigen, or serum is screened for antibodies to that antigen. Preferred methods employ immunoblotting, immunofluorescence, or other immunoassay.

Applicants: Yuan Chang, et al  
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**SCREENING TESTS FOR LYTIC CYCLE ANTIGENS AND  
ANTIBODIES TO KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS**

**Related Application Data**

This is a continuation-in-part of co-pending U.S. application serial number 60/009,267, filed December 27, 1995, which is hereby incorporated herein in its entirety by reference.

**Technical Field of the Invention**

This invention relates to a diagnostic test for Kaposi's sarcoma and/or a serologic test for infection with Kaposi's sarcoma associated herpesvirus (HHV8) in patients, including but not limited to those infected with human immunodeficiency virus (HIV).

**Background of the Invention**

Kaposi's sarcoma (KS), a multifocal vascular tumor, was first described by the Hungarian dermatologist Moritz Kaposi in 1872 (reference 47 in the list following the Examples; subsequent references herein are denoted by a number in parenthesis referring to the same list). Since then four epidemiologic and clinical variants of KS have been distinguished. Classical KS refers to the rare disease recognized in Europe and North America prior to the acquired immune deficiency

- 2 -

syndrome (AIDS) epidemic. Affecting men of Eastern European or Mediterranean heritage, usually in the fifth or sixth decades of life, classical KS is an indolent tumor most often involving the lower extremities (84). In the 1950's an endemic form of KS was recognized in central Africa; endemic KS accounted for up to 10% of neoplasms in adult Ugandan men (8, 58). Endemic KS can be a highly aggressive tumor with considerable lymphatic and visceral involvement (41). As with classical KS, men are affected 10-15 times more often than women, but younger age groups, including children, are also affected. More recently, allograft recipients on immunosuppressive therapy have developed "transplant-associated" KS.

10 Although a rare complication of immunosuppression, KS incidence in renal transplant patients has been estimated to exceed that in the general population by 150 to 200-fold (75). In these patients, the tumor may regress with reduction of immunosuppressive drugs. In the early 1980's a disseminated form of epidemic KS was observed in young homosexual men, and was soon recognized as a sentinel

15 manifestation of AIDS (32, 33). KS is the most common neoplasm in patients infected with HIV.

The possibility that an infectious agent was involved in the pathogenesis of KS was suggested by clustering of the disease in well defined patient populations and the relationship to immunosuppression. Several observations suggested that the putative infectious agent was sexually-transmitted (9). Over 95% of HIV-associated KS cases occurred in men infected with HIV through sex with other men. Among homosexual or bisexual men, the incidence of KS is higher in New York and California than in the Midwest. In addition, there has been a decline in the incidence and prevalence of KS which cannot be easily explained by changes in the demography or geography of the HIV epidemic (24). Furthermore, among women who develop AIDS-associated KS, the mode of HIV infection has most commonly been sexual intercourse with a homosexual or bisexual man (33). In Africa, where HIV is spread primarily through heterosexual intercourse, women are now developing KS in rates approaching those in men (76).

Several neoplasms in HIV-infected patients have been associated with infectious agents. AIDS associated non-Hodgkin's lymphoma is frequently associated with Epstein-Barr virus (EBV) (4, 50, 60, 110) and anogenital cancer with human papilloma virus (HPV) (91). Several agents have been investigated as etiologic factors in KS. Cytomegalovirus (CMV) has been linked to KS by serologic studies (25) and by a large epidemiologic study (35), but efforts to identify CMV genome in KS lesions have yielded inconsistent results (2, 39, 44). Papillomavirus antigens and HPV16 DNA have been detected in some KS biopsies (22). A high prevalence of serum antibodies to *Mycoplasma penetrans* has also been associated with KS (106), but, as with CMV and HPV, it has been difficult to establish a convincing etiologic link.

Recently, Chang, *et al.*, found novel DNA sequences, with significant sequence homology to the gammaherpesviruses Epstein-Barr virus (EBV) and *Herpesvirus saimiri*, in KS lesions (1, 17). The putative new herpesvirus has been designated KS-associated herpes-like virus (KSHV). Sequences of the newly discovered gammaherpesvirus KSHV are detectable in KS tissue from all variant forms of the disease (26, 17, 85, 93, 109). Moreover, the KSHV sequences are found in body cavity lymphoma and in multicentric Castleman's disease (15, 92). These sequences are part of a herpesvirus genome that, at least in certain body cavity lymphoma cell lines, can give rise to virions (63, 65, 80). KSHV genomes have also been found in non-KS skin lesions from renal transplant recipients and in semen from healthy individuals (56, 69, 79).

The discovery of KSHV raises several questions concerning the association of the virus with KS and the distribution of the virus in human population: i) Is the association specific for KS? ii) Does the association represent *de novo* infection or reactivation of a silent latent infection? iii) Is KSHV infection ubiquitous in the human population or distributed only within selected high risk populations?

The development of serological tests to measure antibodies to KSHV should help to clarify the significance of the association of the virus with Kaposi's sarcoma and body cavity lymphoma and aid in diagnosis. Seroepidemiology may also help to determine whether the infection is primary or reactivated. Several  
5 different serological tests employing KSHV-infected body cavity lymphoma cells measure antibodies to antigens of the virus. Two tests measure antibodies to latent nuclear antigens detected by immunofluorescence and immunoblotting (37, 48). The general conclusions from these seroepidemiologic analyses and the results described herein are similar. There is high concordance between presence of  
10 antibodies to KSHV antigens and the presence of KS (36, 37, 48, 68). Antibodies are found less frequently among high risk populations, for example HIV-infected patients without KS, than among patients with KS. Antibody is generally lacking among people who are healthy or at low risk for KS such as HIV-infected hemo-  
15 phorbol-induced lytic cycle antigens detectable by indirect immunofluorescence, has found antibody in about 25% of the American general population (55).

It would be useful to have a screening method such as a serologic test for antibodies to Kaposi's sarcoma-associated herpesvirus, for diagnosis of Kaposi's sarcoma and for infections with the virus that may be associated with a wide  
20 variety of diseases. It would also be useful to have a serologic test for screening blood products.

#### **Summary of the Invention**

It is an object of the invention to provide a clinical test for Kaposi's sarcoma and infections with Kaposi's sarcoma-associated herpesvirus, for diagno-  
25 ses, to follow therapy and to monitor blood products for transfusion.

- 5 -

It is a further object of the invention to provide a diagnostic test that is simple, specific, sensitive, noninvasive, and economical.

5 It is another and more specific object of the invention to provide a serologic test for antibodies to lytic cycle antigens of Kaposi's sarcoma associated herpesvirus or HHV8 (human herpesvirus eight). It is an additional object of the invention to detect expression of Kaposi's sarcoma associated lytic cycle antigens such as small viral capsid antigen, in biological samples from patients.

10 These and other objects are accomplished by the present invention which provides an assay for the detection of antibody or antigen present in biological samples, particularly fluid samples such as serum, to lytic cycle Kaposi's sarcoma associated herpesvirus antigens, *e.g.*, a small viral capsid antigen encoded by the DNA sequence set out in SEQ ID NO 1, DNA that hybridizes with it, and degenerate and complementary sequences. The method of the invention typically involves  
15 obtaining a fluid biological sample from a patient and determining the presence or absence of antibodies to marker antigen in the sample. Observation of antibodies to marker antigen is observed in samples from patients with or without Kaposi's sarcoma.

20 Preferred methods employ immunoblotting, immunoassay, or immunofluorescence to determine the presence of antigen and/or antibody. In some embodiments, for example, immunoblotting or an enzyme-linked immunosorbent assay for qualitative determination of antibodies to marker antigen, which are lytic cycle proteins of Kaposi's sarcoma associated herpesvirus or HHV8. In preferred  
25 embodiments, the antigen is a lytic cycle polypeptide identified as serologic markers for Kaposi's sarcoma associated herpesvirus or HHV8, or, more preferably, the Kaposi's sarcoma-associated herpesvirus small viral capsid antigen described above. Preferred screening tests employ at least one control assay of a sample containing a known amount of antigen and/or a comparison to a sample containing no antigen or antibody.



### Description of the Figures

The figures include photographs of immunoblots illustrating experimental observations more fully described below.

Figure 1 shows specific recognition of KSHV polypeptides in chemically  
5 treated BC-1 cells established from an AIDS-associated body cavity B cell lymphoma. Figure 1A shows reactivity of untreated BC-1 (labelled BCBL) and B95-8 cells with a 1:200 dilution of RM, a reference human antibody to EBV. RM recognizes the EBV polypeptides EBNA1 and p21 in BC-1 cells. Figure 1B shows  
10 reactivity of untreated and chemically treated cells with a 1:400 dilution of serum 01-03 from a patient with KS. Cells were treated with TPA and n-butyrate (B) for 48 hrs. The immunoblots were prepared from 10% SDS polyacrylamide gels. Serum 01-03 recognizes many antigenic polypeptides in chemically treated BC-1 cells.

Figure 2 shows immunoblot detection of KSHV p40 by sera from pa-  
15 tients with KS. Extracts were prepared from BC-1 cells (containing KSHV and EBV) and clone HH514-16 cells (containing only EBV) that were uninduced or treated for 48 hrs with chemical inducing agents, n-butyrate, TPA, or a combination of the two chemicals. Panels A and B show the reactivity of immunoblots prepared from 12% sodium dodecyl sulfate-polyacrylamide gels with 1:200  
20 dilutions of serum from two HIV-1 positive patients with Kaposi's sarcoma.

Figure 3 illustrates detection of KSHV lytic cycle antigens by indirect immunofluorescence. BC-1 cells were untreated (panels a, c, e) or treated with n-butyrate (panels b, d, f) for 48 hrs. Indirect immunofluorescence with a 1:10 dilution of serum from two patients with KS, 04-18 (panel a, b) and 04-38 (panel  
25 e, f) and a serum, 04-37 (panel c, d), from a control patient without KS.

Figure 4 shows selective amplification of EBV and KSHV DNA in BC-1 cells by inducing chemicals. DNA was prepared from HH 514-16 cells and from BC-1 cells that were treated with *n*-butyrate (B), TPA(T), or both chemicals (T/B) for 48 h. The DNA was digested with *Bam*HI and electrophoresed in a 1% agarose gel. A Southern blot was sequentially probed with EBV *Xho*I1.9 which detects the EBV terminal repeats (TRs) (panel A), EBV *Bam* HI Z and KS 631 *Bam* (panel B).

Figure 5 illustrates the effect of phosphonoacetic acid on *n*-butyrate-induced amplification of KSHV DNA. HH514-16 or BC-1 cells were pretreated for 48 hours with medium in the absence of PAA(0) or with PAA(+); inducing chemicals TPA(T) or butyrate (B) were added and DNA prepared after 48 h. Southern blots were probed with EBV *Xho* 1.9 (top) or KS 631 *Bam* (bottom).

Figure 6 shows induction of KSHV lytic cycle mRNAs by *n*-butyrate. Northern blot of cytoplasmic RNA derived from HH514-16 (Cl. 16) cells (lanes 1, 2) or BC-1 clone D5 cells (lanes 4-7). The cells were untreated or treated 48 hours previously with the indicated inducing agents, *n*-butyrate (BUT), TPA, or a mixture. The blot was probed sequentially with KS330 *Bam* (A), EBV-BFRF3 (B), and b actin (C).

Figure 7 shows immunoblot detection of EBV and KSHV polypeptides. BC-1 cells were (-) untreated; (B) *n*-butyrate treated; (T) TPA treated. A) Reactions with three human sera, RM and SJ from healthy EBV-seropositive adults, and 01-03 from a patient with Kaposi's sarcoma. B) Reactions with three monospecific rabbit antisera to EBV lytic cycle products, BZLF1, an immediate early gene product, BFRF3 and BLRF2, late gene products.

Figure 8 shows single-cell clones of BC-1 cells containing both viruses. A) Presence of KSHV and EBV DNA in single-cell clones. Lanes 1 and 16 contain size marker DNA fragments. Cellular DNA from single-cell clones of BC-

1 cells is found in lanes 2-5 and in lanes 9-12. Lanes 2-8 contains PCR reactions for KSHV DNA; a 1851 nt fragment is diagnostic. Lanes 9-15 contains PCR reactions for EBV DNA; a 304 nt fragment is diagnostic. B) Expression of EBV and KSHV polypeptides in three single-cell clones of BC-1 cells. Three clones were  
 5 untreated (-) or treated with sodium butyrate (+) for 48 h. An immunoblot was reacted with an EBV-positive human antiserum (top). The immunoblot was reprobbed with antiserum from a patient with KS (bottom).

Figure 9 illustrates transmission electron microscopy of BC-1 cells. A) General low-power view of an *n*-butyrate treated cell. Many herpesvirus nucleocapsids (arrows), mostly of "b" type, were present in the nucleus (Nu). B) The  
 10 only extracellular morphologically mature particle seen having appearance of a herpesvirus (see text below). C) An extracellular particle lacking an electron dense-core. D) Area of nucleus (Nu) and cytoplasm (Cyt) with marked reduplication of the nuclear envelope (NE) and associated membrane structures (MS). A  
 15 viral nucleocapsid (arrow) was associated with these structures. E, F, G). Higher power views of type "b" and type "c" (arrowhead) nucleocapsids found in the nucleus (Nu) and budding from the nuclear membrane F).

Figure 10 shows detection of EBV DNA but not KSHV DNA in supernatant fluids of *n*-butyrate-treated BC-1 cells. Shown are PCR reactions for b actin  
 20 (331 nt) (lanes 2, 3) KSHV DNA (1851 nt) (lanes 4, 5, 6) and EBV DNA (331 nt) (lanes 7, 8, 9). Markers (M) consisted of lambdaphage DNA *Hind*III digest (lane 1) or *phiX* DNA (*Hae*III digest) (lane 10). "BC-1 supe" (lanes 2, 4, 7) consisted of DNase treated virion preparations from 75 ml of butyrate induced BC-1 cells (see the Examples section below). DNA prepared from the same BC-1 cells (lane 6)  
 25 served as a positive control for the KSHV genome; B95-8 cell DNA (lane 9) was a positive control for EBV DNA. BJAB cell DNA (lanes 3, 5, 8) served as a negative control.

Figure 11 shows detection of EBV DNA but not KSHV DNA in HUCL cells transformed with BC-1 supernatant fluids. Shown are PCR reactions for b actin DNA (panel a), KSHV DNA (panel b) and EBV DNA (panel c). Markers (M), 1 *Hind*III and fX174 *Hae*III are in lanes 1 and 8. Lanes 2-5 contain cellular DNA from human umbilical cord lymphocytes (HUCL) that were transformed by  
5 supernatant fluids from BC-1 cells. The supernatants were from cells that were untreated (lane 2), treated with TPA (lane 3), n-butyrate (lane 4) or a mixture of TPA and n-butyrate (lane 5). BJAB cells (lane 6) were a negative control for KSHV and EBV DNA. BC-1 cells (lane 7) were a positive control for both  
10 viruses.

Figure 12 gives a sequence analysis of the KSHV sVCA gene. (A). Nucleotide and predicted amino acid sequence of sVCA. cDNA sequences are shown in uppercase (and are set out in SEQ ID NO 1); 5' flanking genomic sequences are shown in lowercase. The 170 amino acids of the ORF deduced from  
15 the sequence are depicted beneath each line of nucleotide sequence. The first nucleotide of the cDNA was arbitrarily assigned as position 1. The putative TATA element **tattaa** is shown in bold and the polyadenylation recognition sequence AATAAA is underlined. (B). Amino acid sequence comparison among KSHV sVCA, EBV BFRF3 and HVS ORF65. Sequences were aligned with the GCG  
20 pileup program. The gap-weight is 3.00; and gap-length-weight is 0.1. Percentage similarity/identity between KSHV sVCA versus EBV BFRF3 are 48%/27%; between KSHV sVCA versus HVS ORF65 are 60%/40%.

Figure 13 shows transcription of sVCA in BC-1 cells. (A). sVCA mRNA expressed upon chemical induction of BC-1 cells. U, B, T or B+T represent cells that were untreated, induced with butyrate, induced with TPA, or induced  
25 with butyrate and TPA. No RNA was loaded in lanes 3, 8 and 11. The probe was a PCR amplified product of sVCA ORF labeled with [ $\alpha^{32}$ P]-dCTP by the random-prime method. Quantitation of RNA loading was achieved by hybridizing the same filter with DNA encoding H1 RNA of human RNase P. (B). Sensitivity of sVCA

expression to inhibitors of viral DNA synthesis and protein synthesis. Cells were pretreated with 500 $\mu$ M phosphonoacetic acid (PAA), 500 $\mu$ M phosphonoformic acid (PFA), or 100 $\mu$ M acycloguanosine (ACG) for 10 hours or cycloheximide (100 $\mu$ g/ml) for 6 hours before induction with sodium butyrate for 18 hr. RNAs prepared from each sample were analyzed by Northern analysis.

Figure 14 shows expression of sVCA polypeptide. (A) Expression of sVCA in COS-7 and 293T cells. Cells were transfected with pBK-CMV-sVCA (lanes 1 and 3), with vector control pBK-CMV (lanes 2 and 4). An immunoblot was probed with human serum 0103 from a patient with KS. (B) *In vitro* translation and immunoprecipitation of KSHV sVCA. Lane 1 and 2 represent proteins synthesized in the TNT<sup>®</sup> system from pBK-CMV vector control and pBK-CMV-sVCA. Lanes 3-10 represent immunoprecipitation of the *in vitro* translated product by patient sera. P1 and P2, AIDS patients with KS; P3 Mediterranean KS patient; P4, transplant KS patient; P5, AIDS patient without KS; P6, Mediterranean patient without KS; P7, transplant patient without KS; lane 10, healthy donor. (C) Expression of sVCA in *E. coli*. Lane 1, vector control, pET30b. Lane 2, pET30b-vMIP1, a KSHV-encoded chemokine. Lane 3, pET30b-sVCA. An immunoblot containing bacterial extracts was probed with KS patient serum 0103. (D) Expression of sVCA from KSHV genome in BC-1 cells. Lane 1, 2, 3 and 4 contain extracts from BC-1 cells untreated, induced with butyrate, induced with TPA, induced with TPA plus butyrate. Lane 5 and 6 contain extracts from bacteria transformed with pET30b vector or pET30b-sVCA. An immunoblot was probed with serum from a rabbit which was immunized with purified sVCA expressed in *E. coli*.

Figure 16 illustrates recognition of KSHV sVCA expressed in *E. coli* by sera from KS patients. Extracts from *E. coli* transformed with pET30b or pET30b-sVCA were used to detect specific antibodies by immunoblotting. Sera 0104, 0105, 0106, 0118 and 0438 were from patients with KS. Sera 0107, 0117, 0202, 0205 and 0421 were from patients without KS.

- 11 -

Figure 17 shows purification of KSHV sVCA expressed in *E. coli*. Lane 2, total extracts from *E. coli* with pET30b-sVCA induced by IPTG. Lane 3, soluble supernatant of the total extract. Lane 4, flow-through of Nickel column. Lane 5 to 12 are eluent fractions from the Nickel column after washing (see Example 3).  
5 Fractions E4, E5 and E6 were pooled and loaded on an S•Tag™ agarose column. Lanes 13 to 20 are eluent fractions from S•Tag™ agarose column. The gels were stained with Coomassie blue.

Figure 18 shows screening of human sera for antibodies to purified KSHV sVCA. A Cassette Miniblot™ system was used in the western blot procedure. Eluent fraction E3 from S•Tag™ agarose column was used in panel A. Comparable eluent from *E. coli* transformed with pET30b was used as a negative control antigen in panel B. The same twenty-five sera were tested against purified sVCA (panel A) and control antigen (panel B). The signal representing specific antibody binding to KSHV sVCA is indicated by an arrow. Positive reactions are  
10 seen in lanes 1, 5, 7, 12, 13, 14, 17, and 21. Serum 16 with a high background is not scored as positive.  
15

### Detailed Description of the Invention

This invention is based upon the development of a screening test for antibodies to lytic cycle antigens of human Kaposi's sarcoma associated herpesvirus or HHV8 (human herpesvirus eight) that are diagnostic markers for the infection.  
20

In the practice of this invention, infection with Kaposi's sarcoma-associated herpesvirus is diagnosed by assaying for the presence of antibodies to at least one virus marker antigen in biological samples obtained from a patient, or by assay for the antigen. Typical biological samples are fluid and include, but are not  
25 limited to, serum, saliva, semen, urine, or tissue homogenates such as those obtained from biopsies; preferred samples are serum. Alternatively, infection is

- 12 -

diagnosed by assaying for antigen in serum, semen, or blood cells, e.g., peripheral blood mononuclear cells.

Preferred virus marker antigens are lytic cycle proteins produced in the course of infection either by the host organism or by cloned cell lines, or immunologically reactive fragments of these proteins. In one embodiment, marker antigens are produced by the selective induction of lytic gene expression of KSHV in a cell line infected with EBV and KSHV such as BCBL-1 described below by chemicals such as *n*-butyrate that do not stimulate EBV lytic gene expression. The induction yields antigens associated with KSHV such as p40, p27, p60, or small viral capsid antigen (sVCA) or other unique polypeptides observed by serum antibodies in patients infected with Kaposi's sarcoma-associated herpesvirus. Examples are given hereafter. Thus, this invention also provides the selective induction of expression of one herpesvirus in a cell infected with two herpesviruses.

One particularly preferred embodiment employs an assay for herpesvirus encoded small viral capsid antigen, identified, cloned, and sequenced as described in the Examples that follow. The sequence of cDNA encoding the antigen is set out in Figure 12 and SEQ ID NO 1. A 170-amino acid polypeptide encoded by residues 180 to 689 is compared to related polypeptides in Figure 13 (discussed more fully below).

A biological sample is obtained from a patient, and the level of antibody is then determined in the sample. In most embodiments, this is a qualitative determination. A quantitative estimation and comparisons of antibody levels in relation to values obtained from normal persons may be determined in some embodiments, but are not necessary for a diagnosis of infection with Kaposi's sarcoma-associated herpesvirus. However, estimation of antibody or lytic cycle antigen levels may be used to assess the extent of lytic KSHV replication during different phases of the disease in some embodiments.

Biological samples such as serum may be assayed for antibody to marker antigen using any analytical method known to those skilled in the art, e.g., chemical or bioassays. Preferred methods employ immunoblotting, immunofluorescence or other immunoassay including, but not limited to, Western blots, Northern blots, Northern dot blots, enzyme-linked immunosorbent assays, radioimmunoassays, or mixtures of these. Preferred assays are sensitive and specific, such as radioimmunoassays or enzymeimmunoassays employing polyclonal or monoclonal antibodies to the marker antigen.

By "antibody" is meant an immunoglobulin having a specific amino acid sequence by virtue of which it interacts with virus marker antigen that induced its synthesis in cells of the lymphoid series, or with an antigen closely related to it. Any antibody to Kaposi's sarcoma lytic cycle antigens may be employed in the assays of this invention, and some embodiments employ antibodies to more than one antigen. Antibodies useful in one preferred embodiment of the invention reacts with small viral capsid antigen in the sample.

Antibodies to antigen such as sVCA, or other antigen such as p40, p27, or p60, are generated using conventional techniques (summarized in Varley's *Practical Clinical Biochemistry*, 6th ed., CRC Press, 1988, pp. 112-113). Polyclonal antibodies, for example, are obtained by immunizing intramuscularly, subcutaneously, or intradermally, a rabbit, a guinea pig or a sheep with sVCA, reimmunizing with a booster, and harvesting antibody-containing sera in about 7 to 14 days. Monoclonal antibodies, for example, are obtained by immunizing mice with sVCA, testing for antibody content, hybridizing or fusing spleen cells from an immunized mouse with a plasmacytoma cell line in the presence of polyethylene glycol, culturing the fused cell types so produced, and selecting an appropriate clone.

Lytic cycle virus polypeptides used as antigen for antibody production can be a natural form isolated from tissues or a recombinant form expressed in a transformed or transfected microbiological or other cell line. Isolation and purifica-



tion of proteins provided by either means are by conventional techniques including, for example, preparative chromatographic separations such as affinity, ion-exchange, exclusion, partition, liquid and/or gas-liquid chromatography; zone, paper, thin layer, cellulose acetate membrane, agar gel, starch gel, and/or acrylamide gel electrophoresis; immunological separations, including those using monoclonal and/or polyclonal antibody preparations; and combinations of these with each other and with other separation procedures such as centrifugation and dialysis, and the like.

Preparative schemes for isolation are laborious, typically yielding low amounts of protein. To obtain large quantities, expression of a recombinant form is preferred; an example is given hereafter. DNA sequences encoding lytic cycle proteins such as small viral capsid antigen are identified, cloned, characterized, and sequenced, and the putative amino acid sequences of the polypeptides encoded are determined. This invention thus provides purified and isolated DNA molecules comprising DNA sequences encoding sVCA, purified and isolated DNA sequences which hybridize under stringent conditions with sequences encoding the protein, and degenerate and complementary sequences. Also provided are RNA sequences corresponding to the DNA sequences.

Thus encompassed by this invention are cloned sequences defining lytic cycle antigens of Kaposi's sarcoma associated herpesvirus or HHV8 (human herpesvirus eight) such as sVCA (SEQ ID NO 1), or immunologically reactive fragments thereof, which can then be used to transform or transfect a host cell for protein expression using standard means. Also encompassed by this invention are DNA sequences homologous or closely related to complementary DNA described herein, namely sequences of a genomic DNA clone or cDNA encoding a lytic cycle protein, wherein the noncoding strand of the DNA or cDNA hybridizes under stringent conditions with a genomic DNA or cDNA due to pairing between nucleic acid fragments that have a high frequency of complementary base sequences, and RNA corresponding thereto. In addition to the small viral capsid-encoding se-

quences, DNA encompassed by this invention may contain additional sequences, depending upon vector construction sequences, that facilitate expression of the gene. Also encompassed are sequences encoding synthetic small viral capsid polypeptides exhibiting activity and structure similar to isolated or cloned sVCA, particularly immunologically reactive fragments. These are referred to herein as "biological equivalents."

Because of the degeneracy of the genetic code, a variety of codon change combinations can be selected to form DNA that encodes small viral capsid or other lytic cycle polypeptides of this invention, so that any nucleotide deletion(s), addition(s), or point mutation(s) that result in a DNA encoding the protein are encompassed by this invention. Since certain codons are more efficient for polypeptide expression in certain types of organisms, the selection of gene alterations to yield DNA material that codes for a protein useful in the assays of this invention are preferably those that yield the most efficient expression in the type of organism which is to serve as the host of the recombinant vector. Altered codon selection may also depend upon vector construction considerations.

DNA starting material which is employed to form DNA coding for sVCA and related polypeptides of this invention may be natural, recombinant or synthetic. Thus, DNA starting material isolated from tissue or tissue culture, constructed from oligonucleotides using conventional methods, obtained commercially, or prepared by isolating RNA coding for the polypeptide, and using this RNA to synthesize single-stranded cDNA which is used as a template to synthesize the corresponding double stranded DNA, can be employed to prepare DNA useful for the synthesis of marker antigen to be employed in the practice of this invention. An example is given hereafter.

DNA encoding the proteins of this invention, or RNA corresponding thereto, are then inserted into a vector, *e.g.*, but not limited to, a p series plasmid such as pBR, pUC, pUB or pET, and the recombinant vector used to transform a

microbial host organism. Example host organisms useful in the invention include, but are not limited to, bacterial (e.g., *E. coli* or *B. subtilis*), yeast (e.g., *S. cerevisiae*), mammalian (e.g., mouse fibroblast or other tumor cell line), or insect (e.g., using a baculovirus expression vector). Use of *E. coli* is illustrated in Example 3.

5 This invention thus also provides novel, biologically functional viral and circular plasmid RNA and DNA vectors incorporating RNA and DNA sequences describing sVCA generated by standard means. Culture of host organisms stably transformed or transfected with such vectors under conditions facilitative of large scale expression of the exogenous, vector-borne DNA or RNA sequences and isolation of the

10 desired polypeptides from the growth medium, cellular lysates, or cellular membrane fractions yields the desired products used in the assays for Kaposi's sarcoma-associated herpesvirus.

The present invention thus provides for the total and/or partial manufacture of DNA sequences coding for sVCA, and including such advantageous

15 characteristics as incorporation of codons preferred for expression by selected non-mammalian hosts, provision of sites of cleavage by restriction endonuclease enzymes, and provision of additional initial, terminal or intermediate DNA sequences which facilitate construction of readily expressed vectors. Correspondingly, the present invention provides for manufacture (and development by site specific

20 ic mutagenesis of cDNA and genomic DNA) of DNA sequences coding for microbial expression of sVCA analogues which differ from the forms specifically described herein in terms of identity or location of one or more amino acid residues (i.e., deletion analogues containing less than all of the residues specified for the protein, and/or substitution analogues wherein one or more residues are added to a

25 terminal or a medial portion of the polypeptide), and which share the immunological properties of sVCA described herein.

DNA (and RNA) sequences of this invention code for all sequences useful in securing expression in procaryotic or eucaryotic host cells of polypeptide products having at least a part of the primary structural conformation, and one or

more of the biological properties of sVCA which are comprehended by: (a) the DNA sequences encoding sVCA as described herein, or complementary strands; (b) DNA sequences which hybridize (under hybridization conditions) to DNA sequences defined in (a) or fragments thereof; and (c) DNA sequences which, but  
5 for the degeneracy of the genetic code, would hybridize to the DNA sequences defined in (a) and (b) above. Specifically comprehended are genomic DNA sequences encoding allelic variant forms of p40, and sequences encoding RNA, fragments thereof, and analogues wherein RNA or DNA sequences may incorporate codons facilitating transcription or RNA replication of messenger RNA in non-  
10 vertebrate hosts.

To assay for lytic cycle proteins such as sVCA, one embodiment employs an enzyme-linked immunosorbent assay (ELISA, described in Ausubel, F.M., *et al.*, eds., *Current Protocols in Molecular Biology*, John Wiley, New York, 1990, Unit 11.2.2). ELISAs typically utilize an enzyme such as horseradish peroxidase,  
15 urease, or alkaline phosphatase conjugated to the anti-intestinal lipid binding protein antibody or conjugated with a tag that interacts with a correspondingly tagged antibody. Example tags, where employed, are avidin and biotin. Test serum is incubated in the wells of microtiter plates with conjugated antibody. If the serum contains antigen, the conjugated antibodies adhere to it. Subsequent  
20 measurement of enzyme activity estimates how much tagged antibody is present and bound to the polypeptide. From that, amounts of protein in the original test sample is calculated. Preferred ELISAs employ substrates known to those skilled in the art to be easily measurable, for example, by viewing color development in comparison with standards or by employing a spectrophotometer.

25 Most preferred substrates are chromophoric or yield chromophoric products, so that enzyme activity can be readily measured by the appearance or disappearance of color. Examples of enzyme substrates include p-nitrophenyl phosphate for alkaline phosphatase, bromocresol purple and urea for urease, p-nitrophenyl- $\beta$ -galactopyranoside for  $\beta$ -galactosidase, and the like. Horseradish peroxi-

dase requires hydrogen peroxide in addition to another substrate that serves as a hydrogen donor including, for example, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 5-aminosalicylic acid, o-diaminobenzidine, 3,3'-dimethoxybenzidine, o-phenylenediamine (free base or dihydrochloride), 3,3',5,5'-tetramethylbenzidine (base or dihydrochloride), and the like chromogens.

An alternate embodiment employs a radioimmunoassay (RIA, described in Ausubel, cited above, at § 11.16.1). Typical RIAs employ antigens radiolabelled with  $^{125}\text{I}$ ,  $^3\text{H}$  or other isotope that can be easily detected. For example,  $^{125}\text{I}$ -labelled I-FABP can be employed. Antibody is titrated with labelled antigen, and the activity and sensitivity of the antiserum is determined. A dilution series of samples to which known amounts of antigen have been added are distributed in wells of microtiter plates. Antibody is added, the well material and/or the supernatants analyzed for radioactivity after incubation and compared to a standard curve prepared using pure antigen. Amounts of unlabelled antigen bound are calculated by difference. These and other variations on RIA protocols known by those skilled in the art are encompassed by this invention.

Various other competitive or noncompetitive immunoassay protocols applicable to the measurement of antibodies to the lytic cycle antigens may be employed, including those that involve contacting a sample to a support having already bound thereto either a marker antigen or antibody described above, preferably a lytic cycle antigen such as sVCA. The sample is incubated with the support for a time under conditions sufficient to bind any corresponding antibody or antigen in the sample to the antigen or antibody on the support. Upon subsequent removal of unbound sample from the support, the level of corresponding sample antibody or antigen bound to the support, if any, is estimated directly or indirectly using standard techniques.

This invention further includes diagnostic kits for detecting the presence of antibodies to lytic cycle antigens of Kaposi's sarcoma associated herpesvirus or

HHV8, comprising a container containing antibodies to the lytic cell cycle antigens such as sVCA and the reagents necessary for practicing any one of the assays described above. The kits further comprise antibodies to marker antigen such as sVCA to use as a test control in some embodiments.

5           In one embodiment of the invention, a method for screening for the presence or absence of antibodies to Kaposi's sarcoma associated herpesvirus or HHV8 in a patient comprises obtaining a biological sample, preferably serum, from the patient, estimating the level of antibodies to lytic cell antigens of Kaposi's sarcoma associated herpesvirus or HHV8 such as sVCA in the sample as described  
10 above, and determining the presence of Kaposi's sarcoma associated herpesvirus or HHV8 antibodies by observation of antibodies to marker lytic cell antigens in the sample. At least one control is employed in preferred embodiments, so that the estimated level of antibodies to sVCA or other lytic cycle antigen in the sample is compared with a control sample containing a known amount of antibodies to sVCA  
15 or other known lytic cycle antigen and/or a control sample containing no corresponding antibody to sVCA or other lytic cycle antigen. Another control consists of a comparison of the reactivity of the antibody sample with antigen preparation that contains no corresponding sVCA or other lytic cycle antigen.

          This invention thus provides novel diagnostic methods employing  
20 biochemical markers for detection of antibodies to Kaposi's sarcoma-associated herpesvirus. Because of the importance of the control and diagnosis of AIDS and AIDS-related problems, it is an advantage of the invention that the test can not only be used for diagnosis and differential diagnoses of Kaposi's sarcoma and infections with the virus that may be associated with a wide variety of other  
25 diseases, but it can also be used for therapy monitoring and for blood screening.

          The following examples are presented to further illustrate and explain the present invention and should not be taken as limiting in any regard.

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### Example 1

#### Antibodies to Butyrate-Induced Antigens of Kaposi's Sarcoma-Associated Herpesvirus in Patients with HIV-1 Infection

Patients: Serum was collected from a convenience sample of 48 patients with AIDS and KS and 54 HIV-infected control patients seen at several clinical sites in Connecticut, New York, and California. Demographic and clinical information was recorded on standardized forms which were linked to samples by a numerical code. Ninety nine (97%) were male; 92 of the subjects (90%) were homosexual or bisexual (see Table 1, below). All KS patients were male. In 46 patients the diagnosis was histologically confirmed; in two patients the diagnosis of KS was unequivocal on clinical grounds.

Cell lines: The BC-1 line was established from an AIDS-associated body cavity B cell lymphoma (BCBL) (16,51). KSHV DNA sequences can be detected in BC-1 cells by DNA hybridization with KS 330 *Bam* and KS 631 *Bam*, probes that were originally generated by representational difference analysis (17,57). BC-1 cells also contain an EBV genome detectable with several different EBV DNA probes (16). B95-8 is an EBV producer marmoset cell line that can be efficiently induced into EBV lytic cycle gene expression by phorbol esters (12-O-tetradecanoyl-phorbol-13 acetate, TPA) (67, 111). HH 514-16 is an EBV containing cell clone, originally from a Burkitt lymphoma, that is optimally inducible into EBV lytic cycle gene expression by sodium butyrate (59,78). BL41 is an EBV-negative Burkitt lymphoma cell line (14). B95-8, HH514-16 and BL41 do not hybridize with the KSHV probes. All cell lines were cultured in RPMI 1640 medium containing 8-15% fetal calf serum.

Immunoblotting Assays: Extracts of uninduced BC-1 cells or cells that had been treated with 20 ng/ml TPA (Calbiochem) or 3 mM n-butyric acid, sodium salt (Sigma), or a mixture of inducing chemicals for 48 hrs were prepared by sonication. HH514-16 cells, treated similarly, served to control for antibody reactivity to EBV polypeptides. Each lane of a 10% or 12% polyacrylamide gel



**Table 1. Characteristics of the Study Population.**

		Patient Disease Status		
		<u>With KS</u>	<u>Without KS</u>	<u>p=</u>
	n=	48	54	
5	Male	48	5	ns <sup>a)</sup>
	Female	0	3	
	African American	8	5	ns
	Non-Hispanic White	38	43	
	Hispanic	0	5	
10	Other	2	1	
	Homosexual/Bisexual	44	48	ns
	Injection Drug User	0	2	
	Heterosexual	2	3	
	Other/Unknown	2	1	
15	CD4 cells/mm <sup>3</sup>			ns
	0-100	28	21	
	100-300	12	16	
	>300	7	16	
	Unknown	1	1	

20 <sup>a)</sup> Chi square and Fisher's exact analyses revealed no significant (ns) differences between subjects with and without KS.

25 was loaded with extract of  $2.5 \times 10^5$  cells in sodium dodecylsulfate (SDS) sample buffer; electrophoresis, transfer to nitrocellulose and blocking with skim milk followed standard protocols (101). Sera were screened at 1:100 dilution. The reaction was developed by 1.0  $\mu$ Ci of <sup>125</sup>I Staphylococcal protein A (Amersham). Radioautographs were exposed to film for 24-48 hrs. Immunoblotting assays were performed and interpreted on coded sera.

30 Immunofluorescent assay: The antigens being monitored were present in BC-1 cells that were untreated or treated with 3 mM n-butyrate for 48 hrs. Cells were dropped onto slides that were then fixed in acetone and methanol. Sera were

- 23 -

tested at 1:10 dilution, followed by a 1:30 dilution of fluoresceinated sheep anti-human Ig (Wellcome). The reactivity of a serum was compared on untreated and n-butyrate treated BC-1 cells. Untreated BC-1 cells expressed 0.5% to 2.0% antigen positive cells using different sera. Reactivity with 10-20 fold more n-butyrate treated BC-1 cells than untreated cells was considered a positive reaction. Sera containing antibodies to EBV but not to KSHV recognized the same number of antigen positive cells in untreated and n-butyrate treated preparations. All immunofluorescence tests were performed on coded sera. The readers were blinded to disease status or results of immunoblot assays.

10        Chemical induction of KSHV-associated proteins in BC-1 cells: Since sera from HIV-1 infected patients with or without KS would be expected to contain antibodies to EBV polypeptides, and since BC-1 cells are dually infected with KSHV and EBV, it was essential to distinguish EBV polypeptides from those encoded or induced by KSHV. The immunoblot technique was employed to  
15 determine whether BC-1 cells expressed antigenic polypeptides specific for KSHV infection. Figure 1A shows that BC-1 cells expressed at least two EBV polypeptides, representing the latent nuclear antigen EBNA1 and p21, a late capsid antigen complex (105), that were present in other EBV producer cell lines, such as B95-8 (Figure 1A) and HH514-16 (Figure 1B and 2). When sera from patients with KS  
20 were used as a source of antibody in immunoblot reactions with extracts from untreated BC-1 cells, they failed to identify additional antigenic polypeptides that were not also seen in the EBV producer cell lines. However, if extracts were prepared from BC-1 cells that had first been treated with a combination of TPA and butyrate, KS patient sera now recognized a number of novel polypeptides that  
25 were present in the BC-1 cell line but not in EBV producer cell lines (Figure 1B and Figure 2). The molecular weights of the most prominent of these many polypeptides were estimated at about 27 KDa, 40 KDa and 60 KDa on 10% polyacrylamide gels. These polypeptides were detected within 24 hours after addition of the chemical inducing agents. Further experiments showed that n-butyrate was the chemical agent primarily responsible for induction of p40, whereas  
30 p60 could be induced by TPA or n-butyrate. Since p27, p40 and p60 were not

detected in untreated cells and appeared after treatment with chemicals they were thought likely to represent lytic cycle rather than latent cycle polypeptides of KSHV.

p40 and p60 are KSHV specific: Figure 1B and Figure 2 show that  
5 antigenic polypeptides corresponding in molecular weight to p40 were not observed in two EBV producer lines, B95-8 and HH514-16, that were induced into the EBV lytic cycle by the same chemicals. Nor was p40 detected in comparably treated EBV-negative BL41 cells. Many KS patient sera diluted past their reactivity with EBV polypeptides still recognized KSHV-associated p40. Furthermore, n-butyrate  
10 strongly induced expression of p40 in BC-1 cells but had little or no effect on the level of expression of the EBV early or late antigens in the same cells as detected with monospecific antibodies to EBV gene products. Thus, p40 appeared to represent specific expression of the KSHV genome in the chemically induced BC-1 cells. In related experiments it was found that n-butyrate also induced an increase  
15 in the abundance of KSHV DNA and KSHV late lytic cycle mRNAs while having little or no effect on the content of EBV DNA or EBV late cycle mRNAs. TPA, by contrast, induced the EBV lytic cycle in BC-1 cells efficiently; treatment with TPA increased the abundance of EBV DNA while causing only minimal induction of KSHV DNA (70). These findings suggested that the latency to lytic cycle  
20 switch of the two gamma herpes viruses carried by BC-1 cells was under separate control and provided further evidence that the p40 complex observed after butyrate treatment was specific to the KSHV genome.

p40 as a serologic marker for KSHV: While a few highly reactive sera, such as KS 01-03, (Figure 1B; Figure 2D) recognized multiple antigenic proteins  
25 unique to the chemically induced BC-1 cells, including p27, p40 and p60, sera from other patients with KS did not react with p27 or p60 but still recognized p40 (Figure 2). Therefore recognition of p40 was investigated as a serologic marker for infection with KSHV. Sera from 102 HIV-1 infected patients from Connecticut, New York and California were examined for presence of antibodies against p40  
30 (Table 2 below).

**Table 2.** Prevalence of Antibody to KSHV p40 in HIV-1 Positive Patients with and without Kaposi's Sarcoma.

		<u>Patient Disease Status</u>			
5	<u>State of Residence</u>	<u>with KS</u>	<u>(%)</u>	<u>without KS</u>	<u>(%)</u>
	Connecticut	10/14 <sup>a)</sup>	(71)	0/13	(0)
	New York	15/23	(65)	3/28	(11)
	California	7/11	(64)	4/13	(31)
10	Total <sup>b)</sup>	32/48	(67)	7/54	(13)

<sup>a)</sup> Number patients with antibody to p40/Number patients studied.

<sup>b)</sup> The odds ratio for the comparison of prevalence in subjects with and without KS is 13.43 (95 percent confidence interval, 4.48 to 42.0).

15 Thirty two of forty eight patients with KS (67%) had antibodies to p40; only 7 of 54 patients (13%) without KS had antibodies to p40 ( $p < 0.0001$  by Chi square). These seven patients were homosexual or bisexual men from New York City or San Francisco. None of the 13 HIV-positive patients without KS from Connecticut recognized p40. The positive and negative predictive values of the serologic marker for the presence of KS were 82% and 75% respectively. Table 3  
20 below compares the patients with KS whose serum did or did not contain antibodies to KSHV p40. Neither CD4 cell number nor the extent of KS disease predicted the presence or absence of a serologic response to p40.

25 Immunofluorescence assays: Immunoblot assays showed that n-butyrate induced expression of KSHV lytic cycle polypeptides in BC-1 cells without substantially affecting expression of EBV polypeptides (Figures 1B and 2). Therefore it may be that n-butyrate might also induce many more BC-1 cells to switch into the KSHV replicative cycle than into the EBV lytic cycle. Using indirect immunofluorescence with a reference human antiserum that contains antibodies to EBV but not to KSHV (RM, Figure 1A), there were 2% antigen positive cells in

**Table 3.** Comparison of KS patients With and Without Antibody to KSHV p40.

		<u>Patient Serologic Status</u>		
		<u>p40+</u>	<u>p40-</u>	<u>p=</u>
	n =	32	16	
5	African American	7	1	ns <sup>a)</sup>
	White	25	13	
	Hispanic	0	0	
	Other	0	2	
10	Homosexual/Bisexual	29	15	ns
	Heterosexual	2	0	
	Other/Unknown	1	1	
15	CD4 cells/mm <sup>3</sup>			
	0-100	17	11	ns
	100-300	9	3	
	>300	6	1	
	Unknown	0	1	
	Limited <sup>b)</sup> KS	22	9	ns
	Extensive <sup>c)</sup> KS	10	7	
	Biopsy Confirmed	30	16	ns

20 <sup>a)</sup> Chi square and Fisher's exact analyses revealed no significant (ns) differences between subjects with and without KS.

<sup>b)</sup> Limited: confined to skin or lymph nodes or with minimal mucosal involvement.

25 <sup>c)</sup> Extensive: widespread mucosal or visceral involvement or cutaneous disease with significant edema or ulceration.

the untreated BC-1 line and a similar number of antigen positive BC-1 cells that had been treated with n-butyrate. Serum 01-03 that is EBV-positive and highly reactive to KSHV antigens (Figure 1B) detected 2% antigen positive cells in the untreated BC-1 population, presumably the EBV expressing cells, while it detected 30 30% to 50% antigen positive BC-1 cells that had been treated with n-butyrate. The

antigens detected were mainly cytoplasmic and on the cytoplasmic membrane (Figure 3). Other antibody-positive KS case sera recognized a 10-20 fold increase in antigen positive cells after butyrate stimulation. This increase in the number of antigen positive BC-1 cells among the n-butyrate treated population served as the basis of an immunofluorescence screening assay (IFA) for antibodies to inducible KSHV antigens.

The results of the IFA were nearly identical to the immunoblotting assay (Table 4). 64% of patients with KS and 13% of HIV-1 infected patients without KS were reactive by IFA. Only 3 among the 102 sera tested (3%) were discordant in the two assays. One serum scored positive by IFA and negative by immunoblotting; two were considered positive by immunoblotting and negative by IFA (Table 5).

The odds ratio for the association of these antibodies with KS were 13.43 (95% confidence interval 4.5-42) for the immunoblot assay and 12.24 (95% confidence interval 4.1-38) for IFA. The predictive value of a positive test for antibodies using either of the two assays was 82%. Thus, antibodies in sera of

**Table 4.** Prevalence of Antibody Detectable by Indirect Immunofluorescence to KSHV (Antigens in n-Butyrate Induced BC-1 Cells in HIV-1 Positive Patients with and without Kaposi's Sarcoma).

State of Residence	<u>Patient Disease Status</u>			
	<u>with KS</u>	<u>(%)</u>	<u>without</u>	<u>KS (%)</u>
Connecticut	10/14 <sup>a)</sup>	(77)	0/13	(0)
New York	15/23	(65)	3/28	(11)
California	6/11	(67)	4/13	(31)
Total <sup>b)</sup>	31/48	(64)	7/54	(13)

<sup>a)</sup> Number patients with antibody/Number patients studied.

<sup>b)</sup> The odds ratio for comparison of prevalence in subjects with and without KS is 12.24 (95 percent confidence interval 4.11 to 38.0).

Table 5. Concordance of Immunoblotting and Immunofluorescence Assays.

	<u>Patients with KS</u>		<u>Patients without KS</u>			
	<u>IFA+</u>	<u>IFA-</u>	<u>IFA+</u>	<u>IFA-</u>		
5	<u>p40+</u>	30	2	<u>p40+</u>	7	0
	<u>p40-</u>	1	15	<u>p40-</u>	0	47

HIV-1 infected individuals to chemically induced KSHV associated antigens were strongly correlated with the clinical presence of KS.

The findings indicate that KSHV is infrequent but associated with a high rate of apparent disease. In related studies, antibodies to p40 among 45 HIV-  
 10 negative sera tested were not detected. Only 13% of HIV-1 infected patients without KS had antibodies to the KSHV antigens; by contrast a very high proportion of HIV-1 infected men who had clinically evident KS were seropositive. These findings suggests that a high proportion of individuals who are dually  
 15 infected with HIV-1 and KSHV develop KS.

The serologic studies provide a strong correlation between the presence of antibodies to KSHV lytic cycle gene products and clinical KS. Using sera from HIV-1 infected patients Moore, *et al.*, have detected nuclear antigens in uninduced HBL-6 cells, another KSHV and EBV infected cell line derived from the same  
 20 body cavity B cell lymphoma as BC-1 (70). After absorption with EBV producer cells the geometric mean titer of antibodies in sera from 14 patients with KS was nine-fold higher than in 16 patients without KS. These findings also suggest that antibodies to KSHV correlate with development of clinical disease but they imply that sera from HIV-1 infected patients without KS may contain low titer antibodies  
 25 to certain KSHV antigens.

There are two groups of patients described herein whose serologic results require further explanation. One group consists of the few patients with positive serology for KSHV p40 without clinical KS. All seven patients with antibodies to

- 29 -

p40 were gay men from New York City or San Francisco who are at higher risk for KS than the general North American population (33,76). Two of the patients from New York City who were initially seropositive without KS subsequently developed KS; one had gastrointestinal involvement and one cutaneous lesions. It is possible that the visceral lesions may have been present at the time of the initial evaluation.

The other group is the approximately 30% of patients with KS whose sera lacked antibody to p40. The patients with KS who were p40 seronegative were not misclassified since the diagnosis was confirmed in all of them by biopsy (Table 3). p40 may be of low abundance and not stimulate an immune response in some patients. The antibodies being measured may wax and wane with time following infection. If antibody to p40 reflects the extent of lytic KSHV replication, the appearance of these antibodies may vary during different phases of the disease. In some individuals serum antibodies to p40 may be consumed in immune complexes with p40 antigen in the circulation. Although lack of a serologic response to p40 could also reflect severely impaired humoral immunity, no association was made between the degree of immunosuppression, as monitored by the number of CD4 cells, and the presence or absence of antibody to p40 among patients with KS (Table 3).

20

### Example 2

#### Selective Switch between Latency and Lytic Replication of Kaposi's Sarcoma Herpesvirus and Epstein-Barr Virus in Dually Infected Body Cavity Lymphoma Cells

Cell cultures. BC-1 cells were cultured in RPMI medium plus 15 to 20% fetal bovine serum. HH514-16 (Cl.16) cells, in which the P3J- HR-1 strain of EBV is tightly latent but highly inducible by butyrate, were grown on RPMI plus 8% fetal bovine serum (78). All cultures contained penicillin and streptomycin (50 units/ml) and amphotericin B (1 m g/ml).



- 30 -

Cloning of BC-1 cells. Suspensions, containing 100, 10, 1 and 0.5 BC-1 cells per 0.1 ml of RPMI medium with 15% FBS and antibiotics, were placed into 96 well plates covered with a confluent monolayer of MRC-5 cells. The plates were incubated in a humidified 5% CO<sub>2</sub> atmosphere at 36°C. At day 7 after plating all wells containing 100 and 10 cells showed growth while no growth was seen in wells containing 1 or 0.5 cells. The plates with 100 and 10 cells per well were discarded. At day 14 cell growth was observed in 16/96 wells originally plated with 1 cell and in 2/44 wells plated with 0.5 cell. When the BC-1 cells had multiplied sufficiently they were transferred to 24 well plates with MRC-5 cells and then to 25 cm<sup>2</sup> flasks without a feeder layer. Fifteen clones, all derived from the 1 cell/well dilution, were propagated free of feeders.

Chemical induction. BC-1 cells were subcultured into 50 ml volumes and inducing agents were added 24 to 48 h later. TPA was added to a final concentration of 20 ng/ml, and n-butyrate (Sigma 5887) was added to a final concentration of 3mM. One flask was left untreated as a control. The cells were exposed to inducing agents for 24 to 48 h.

Effect of phosphonoacetic acid (PAA). 24 h after cells were subcultured they were resuspended in medium containing 0.4 mM PAA or in medium without PAA. Chemical inducing agents (TPA or n-butyrate) were added 48 h later; cells were harvested for analysis of viral DNA content 48 h after addition of chemical inducing agents.

Immunoblotting assays. Cells, suspended in SDS sample buffer at a concentration of 5 x 10<sup>7</sup> cells/ml, were sonicated and boiled prior to electrophoresis through a 10% polyacrylamide gel. Each lane was loaded with 10 ml of antigen. After electrophoresis the gels were transferred to nitrocellulose at 0.4mA for 2 h and blocked overnight with 5% skim milk. Identical blots were probed with 1:100 dilutions, in skim milk, of three different human sera, with three monospecific rabbit antisera prepared against the EBV gene products BZLF1, an immediate early product, and BLRF2 and BFRF3, late gene products (88.105). The human sera were SJ (EBV+/KSHV-), RM (EBV+/KSHV-) and 01-03 (EBV+/KSHV+).

- 31 -

Antigen antibody reactions were detected by addition of 1mCi of  $I^{125}$  protein A in 10 ml. Blots were exposed to XAR film to produce an autoradiograph.

Immunofluorescence assay. BC-1 cells were treated with inducing agents for 48 h, washed once with PBS, resuspended at  $5 \times 10^5$ /ml in PBS, and dropped  
5 onto microscope slides. When dry, the cells were fixed in acetone: methanol, 2:1, for 5 min and stored at  $-20^\circ\text{C}$ . The cells were stained by indirect immunofluorescence with human patient sera at 1:10 followed by fluorescein conjugated sheep anti-human immunoglobulin (Wellcome MF01) at 1:30. The human sera were from three donors: one was EBV+/KSHV-; one was EBV+/KSHV+; and one was  
10 EBV-/KSHV-.

Southern blot analysis for KSHV and EBV DNA. To prepare total cellular DNA  $2 \times 10^7$  cells from each experimental group were washed once in phosphate buffered saline with no  $\text{Mg}^{2+}$  or  $\text{Ca}^{2+}$ , and resuspended in 5 ml containing 0.2 M Tris, 0.1 M EDTA, 1mg/ml pronase, and 1% SDS. The samples were incubated at  
15  $60^\circ\text{C}$  for 2 h. 1.0 ml of 5M potassium acetate was added and the samples were held on ice for 30 min. The samples were centrifuged for 20 min at 8000 rpm at  $2^\circ\text{C}$  and the supernatants were harvested. RNase was added to a final concentration of 20 mg/ml. The DNA was precipitated by addition of two volumes of 95% ethanol; the pellets were dried and resuspended in 500  $\mu\text{l}$  of 0.01M Tris, pH8, 1  
20 mM EDTA. The concentration of DNA was estimated by OD 260. For Southern analysis 5  $\mu\text{g}$  of cellular DNA was digested with *Bam*HI and electrophoresed in a 1% agarose gel. The gel was transferred to nitrocellulose by the Southern method. DNA probes, labeled with alpha  $^{32}\text{P}$  dCTP by the random prime method, contained about  $10^7$  cpm. The EBV probes were *Bam*HI Z or *Xho*I 1.9; the KSHV probes  
25 were KS 631 *Bam* or KS 330 *Bam*. All probes were excised from their vectors. The hybridized blots were exposed to XAR film overnight. To compare the relative inducibility of the two viral DNAs serial two-fold dilutions of cellular DNA were prepared, beginning at 5  $\mu\text{g}$ , digested with *Bam*HI and analyzed by Southern blot hybridization.

30 Polymerase chain reaction (PCR) analysis for KSHV and EBV DNA. The primers used to amplify KSHV DNA were from a region of KSHV DNA that

- 32 -

encompasses KS 330 *Bam*. The expected amplified product is 1851 bp. The sequences of the KSHV primers were 5' CGGAATTCCTGCGAGATAATTCC-CACGCCGGTC (SEQ ID NO 2) and 5' CGGGATGCAGAACAGGGCTAGGTA-CACACAATTTTCAAG (SEQ ID NO 3). The primers used to amplify EBV DNA  
5 from the EBV BMLF1 ORF produce a product of 304 bp. The two primers were 5'CACCACCTTGTTTTGACGGG (SEQ ID NO 4) and 5' GTCAACCAAC-AAGGACACAT (SEQ ID NO 5). Each reaction contained 200-300 ng of total cell DNA, 100 ng of each primer and 2.5 units of Taq polymerase. The PCR  
10 conditions were 1 cycle at 94°C for 3 min; 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; 1 cycle at 72°C for 10 min. Aliquots of each reaction were electrophoresed in a 1% agarose gel which was stained with ethidium bromide. The PCR products were also detected by Southern analysis.

Northern analysis. Cells were lysed in 150 mM NaCl, 10mM Tris pH8.0, 0.1% NP40 and the nuclei were deposited by centrifugation. RNA was isolated in  
15 7M urea, 350 mM NaCl, 10mM Tris pH 7.5 and 20 mM EDTA. The RNA was electrophoresed in a 1% agarose/6% formaldehyde gel in 20 mM MOPS pH7. Each lane received RNA from  $2.5 \times 10^6$  cells. The gel was transferred to Nytran (S and S) and hybridized with a probe radiolabelled with alpha <sup>32</sup>P dCTP by the random prime method. Probes were derived from an EBV late gene BFRF3, a 754  
20 bp fragment representing the start of the open reading frame, and KS 330 *Bam* (17, 105). RNA loading equivalence was estimated by probing with a 1.8 kbp fragment of the  $\beta$  actin cDNA.

Transmission electron microscopy. Cell pellets from BC-1 cells that were untreated or treated for 48 h with n-butyrate were fixed in glutaraldehyde and  
25 osmium tetroxide and embedded in Epon as described (98). Ultrathin sections were viewed in a Philips EM400T electron microscope at direct magnifications of 17,000X to 60,000X.

Examination of extracellular viral genomes for DNase sensitivity. 75 ml of BC-1 cells at  $10^6$  per ml were treated with n-butyrate for 48 h. The cells were  
30 deposited by two low speed centrifugations at 5K for 5 min, and the supernatant fluid was centrifuged at 35K rpm for 30 min at 4°C in the SW41 Beckman rotor.

- 33 -

Viral pellets were resuspended in 0.5 ml of PBS, 10 mM MgCl<sub>2</sub> and 50 µg/ml DNase (Worthington) and incubated at 37°C for 3 h. NP40 was added to a final concentration of 1% and incubation continued at room temperature for 5 min. 110 m g/ml DNase was added and incubation continued at 37°C for 2 h. The incubation buffer was adjusted to contain 0.1 M NaCl, 50mM Tris pH8.0, 25mM EDTA to inactivate the DNase. Pronase was added to a final concentration of 140 m g/ml and incubation continued overnight at 50°C. The mixture was extracted once with phenol and once with phenol/chloroform and the DNA precipitated with ethanol. The DNA was solubilized in 100 ml 10 mM Tris pH8/1 mM EDTA. 2 µl of DNA was used in each PCR reaction for detection of EBV and KSHV DNA.

Assay for infectivity in BC-1 cell supernatants. 10 ml BC-1 cell cultures at 10<sup>6</sup>/ml were treated with TPA, n-butyrate, a mixture of TPA and n-butyrate or left untreated for 24 h. The cells were washed and resuspended in fresh culture medium. After 48 h additional incubation at 37°C the cells and fluids were frozen and thawed three times. The suspension was centrifuged at 1000 rpm for 5 min, and the supernatant was centrifuged again. Two ml of supernatant was added to 8 ml of human umbilical cord lymphocytes (HUCL) at 10<sup>6</sup> cells/ml. The cells were morphologically transformed after 2 weeks. Three months after infection cellular DNA from each culture and analyzed for KSHV and EBV DNA by PCR.

20

## RESULTS

Selective amplification of EBV and KSHV DNA in BC-1 cells treated with inducing chemicals. To determine whether the two gammaherpesviral genomes harbored by BC-1 cells were under coordinate or separate control, the cells were treated with chemical inducing agents known to effect a switch between latency and lytic cycle gene expression of EBV. TPA and n-butyrate were added singly and in combination; the effect of this treatment on the content of EBV and KSHV DNA was monitored by Southern analysis. For comparison, HH514-16 (Cl.16) cells, in which EBV is tightly latent but highly inducible by n-butyrate, were studied in parallel.

25

EBV underwent spontaneous lytic replication in BC-1 cells. There was a ladder of restriction fragments of heterogeneous size that contain the EBV terminal repeats (Figure 4, panel A, lane 5). This ladder is characteristic of linear EBV DNA destined for encapsidation (49, 77). Treatment of BC-1 cells with TPA  
5 caused an increase in the abundance of EBV DNA as evidenced by an increased signal from the ladder of terminal repeats (Figure 4A, lane 7) or from an internal region of EBV DNA detected with the *Bam*HI Z probe (Figure 4B, lane 7). However, surprisingly, treatment with the combination of TPA and n-butyrate did not increase EBV DNA content in BC-1 cells.

10 HH514-16 (Cl. 16) cells, in which EBV was tightly latent, lacked the ladder of terminal repeats; instead only a single restriction fragment representing the fused termini was seen in untreated cells (Figure 4, lane 1). In Cl.16 cells n-butyrate by itself or in combination with TPA caused an increase in the abundance of EBV DNA (Figure 4, lanes 2 and 4). TPA alone had little or no effect on EBV DNA  
15 content in Cl.16 cells. Thus the response of the EBV genome to chemical induction differed between the two cell lines. In BC-1 cells EBV was induced by TPA, while in Cl.16 cells EBV was induced by n-butyrate.

In BC-1 cells n-butyrate alone, or in combination with TPA, caused an increase in KSHV DNA content (Figure 4B, lane 6 and 8). The content of the  
20 KSHV genome in BC-1 cells was minimally affected by TPA (Figure 4B, lane 7). The same results were obtained whether the blots were probed with KS 631 *Bam* (Figure 4) or with KS 330 *Bam*. These results indicated that DNA amplification of the two gammaherpesviruses carried by BC-1 cells was under separate control. TPA preferentially stimulated EBV DNA replication, while n-butyrate triggered an  
25 increase in KSHV-specific DNA.

Effect of phosphonoacetic acid. Treatment with PAA, a compound that predominantly affects lytic cycle herpesvirus DNA polymerase (95), reduced the level of KSHV DNA in BC-1 cells treated with n-butyrate (Figure 5, compare lanes  
9 and 12). PAA also inhibited spontaneous and TPA-induced EBV DNA synthesis  
30 in the same cells (Figure 5, compare lanes 7 and 10, and lanes 8 and 11). Titration experiments showed that PAA caused about a two-fold inhibition of chemically

- 35 -

induced amplification of KSHV and EBV DNA in BC-1 cells. For comparison, treatment of Cl.16 cells with PAA produced a four-fold inhibition of n-butyrate induced EBV DNA amplification (Figure 5, lanes 3 and 6). These results are consistent with the idea that increases in the DNA content of both gammaherpesviruses were mediated by viral DNA polymerases.

Magnitude of induction. To quantitate the magnitude of the effects of chemical induction on the content of KSHV and EBV DNA, the DNA samples from chemically treated cells were serially diluted and compared with DNA from untreated cells. In the samples from the experiment depicted in Figure 5, TPA induced a 4-fold increase in EBV DNA content in BC-1 cells while the content of KSHV DNA was increased less than 2-fold. Butyrate induced an 8 to 16-fold increase in KSHV DNA content and less than a two-fold increase in EBV DNA content. For comparison, in Cl.16 cells n-butyrate induced an 8 to 16-fold increase in EBV DNA content and TPA did not alter the abundance of EBV DNA. Thus, the level of inducibility of KSHV DNA in BC-1 cells by butyrate was comparable to the magnitude of induction of EBV DNA in the highly inducible Cl.16 cells.

Induction of KSHV lytic cycle mRNAs by n-butyrate. To determine whether inducing chemicals promoted lytic cycle gene expression of the two gammaherpesviruses in BC-1 cells, northern blots were hybridized with probes representing genes encoding capsid components of KSHV and EBV (Figure 6). BC-1 subclone D5, treated with n-butyrate, or a combination of TPA and n-butyrate, expressed a prominent 2.0 Kb mRNA and a less abundant 6.9 Kb mRNA that were detected by KS330 *Bam*, a region homologous to a gammaherpesviral capsid protein (Figure 6A, lanes 5 & 7). These two mRNAs were not identified in untreated BC-1 cells (Figure 6A, lane 4) and were present only in trace amounts in BC-1 cells that had received TPA alone (Figure 6A, lane 6). Although KS 330 *Bam* has homology with EBV, this probe did not detect EBV mRNAs in Cl.16 cells that were induced into the lytic cycle (Figure 6A, lanes 1, 2).

BC-1 treated with TPA expressed three prominent EBV mRNAs of 0.9 Kb, 3.3 Kb, and 4.1 Kb, and a less prominent 3.65 Kb mRNA, all of which were detected by a probe for BFRF3, which encodes a p21 capsid component (Figure

6B, lane 6). Recent studies have shown that only the 0.9 Kb mRNA is a true late transcript; the other mRNAs are expressed early (88, 105). Traces of these EBV lytic cycle mRNAs were detected in untreated or n-butyrate treated BC-1 cells (Figure 6B, lane 7).

5           Whenever n-butyrate was present in BC-1 cultures the cellular b actin signal was markedly diminished (Figure 6C, lanes 5, 7). In the same RNA samples in which there was no detectable cellular  $\beta$ -actin mRNA the KS viral mRNAs detected by the KS 330 *Bam* probe were abundant (Figure 6A, lane 5, 7). A titration of n-butyrate showed that the 3 mM dose of n-butyrate, which maximally  
10 induced the 6.9 Kb and 2.0 Kb mRNAs detected by KS 330 *Bam*, caused loss of detectable steady state mRNA for cellular b actin.

The presence of n-butyrate in combination with TPA in BC-1 cultures was also associated with inhibition of EBV lytic gene expression. The EBV mRNAs present in BC-1 cells treated with TPA were not detected after treatment with a  
15 combination of TPA and n-butyrate (Figure 6B, lane 7). This result mirrored the failure to detect amplification of EBV DNA in BC-1 cells treated with a combination of TPA and n-butyrate (Figure 4A, lane 8). However, in Cl.16 cells the combination of TPA and n-butyrate did not result in inhibition of either EBV late  
20 gene expression (Figure 6B, lane 2) or cellular  $\beta$ -actin expression (Figure 6C, lane 2). This differential sensitivity of KSHV, EBV and host cell mRNA expression to the action of n-butyrate is consistent with the hypothesis that KSHV lytic gene expression is associated with a mechanism that shuts off host cell and EBV mRNA expression.

Comparing induction of EBV and KSHV lytic cycle polypeptides. BC-1  
25 cells spontaneously expressed both latent and lytic cycle products of EBV. Two polypeptides, one about 95 kD, corresponding to EBNA1, and the other p21, a late capsid antigen complex encoded by two EBV genes BFRF3 and BLRF2 (88), were detected on immunoblots by human antisera to EBV (Figure 7A). The abundance of the p21 complex was minimally altered by n-butyrate treatment and induced  
30 about 5-fold by treatment with TPA. Replicate immunoblots (Figure 7B) probed with monospecific antisera showed that the EBV lytic cycle products encoded in

- 37 -

the BZLF1, BFRF3 and BLRF2 open reading frames were all selectively induced by TPA and relatively unaffected by n-butyrate.

To determine whether BC-1 cells also expressed polypeptides related to KSHV, either spontaneously or following chemical induction, sera from patients with Kaposi's sarcoma were used as a source of antibody on immunoblots. Some of the most reactive of these human antisera (Figure 7A) detected at least three polypeptides, p27, p40, and p60 in chemically treated BC-1 cells. p27 and p40 were maximally induced by n-butyrate treatment. p60 was induced to a similar extent by TPA and by n-butyrate. These proteins were not detected by reference human antisera containing antibodies to EBV early and late antigens.

p27, p40 and p60 were not detected by KS patient sera in EBV-infected producer cell lines, HH514-16 or B95-8, that were maximally induced into the lytic cycle. Nor were the proteins present in EBV-negative cells such as BJAB or BL41 which lack both KSHV and EBV genomes. Thus this group of proteins was specific to chemically induced BC-1 cells. Their pattern of induction by chemicals was parallel to the induction of KSHV DNA and mRNA in BC-1 cells (Figures 4 to 6). Therefore these polypeptides are likely to be encoded by KSHV or, less likely, to be cellular proteins that are induced during the KSHV lytic cycle.

Presence and expression of both KSHV and EBV genomes in single cell clones of the BC-1 cell line. Differential induction of EBV and KSHV expression in BC-1 cells could result from the differential response to chemical induction of two different populations of cells, one infected with KSHV and the other with EBV. Alternatively there could be two populations of BC-1 cells, both dually infected with KSHV and EBV, which might differ in their response to chemical induction. To explore these possibilities, single cell clones of BC-1 cells were obtained by limiting dilution. Each of the 15 clones analyzed contained both genomes (Figure 8A). EBV spontaneously replicated in all clones. Each clone expressed the latent EBV nuclear antigen 1 and the EBV late lytic replicative p21 complex (Figure 8B). The abundance of EBV p21 was unaffected by n-butyrate treatment. However, in all clones butyrate treatment induced the p40 polypeptide detected with KS patient antiserum (Figure 8B bottom). There was variability



- 38 -

among the clones in the level of p40 expression; e.g., clone D5 expressed more of this antigen than did clone D10.

Cell by cell assays for EBV and KSHV lytic cycle antigens. The increased expression of p40 and other KSHV polypeptides induced by butyrate could result from amplified levels of viral polypeptide expression within a few cells that are spontaneously in the KSHV lytic cycle or could be due to recruitment of additional cells to enter the lytic cycle. To distinguish these possibilities the proportion of cells expressing lytic cycle EBV and KSHV antigens was measured by indirect immunofluorescence assays.

Human EBV antibodies or monospecific antibodies to EBV capsid antigen components, such as BLRF2, detected EB viral capsid antigen (VCA) in 1% to 2% of BC-1 cells. Human antisera and monoclonal antibodies to EBV early antigens detected a similar proportion of antigen positive cells. The percentage of cells expressing EBV lytic cycle products increased about 5-fold after treatment with TPA but was not affected by n-butyrate. Thus, the EBV genome was latent in about 98% of BC-1 cells and remained so in 90% of the cells, even after TPA treatment.

Results of immunofluorescence reactions were dramatically different when sera from patients with KS were the source of antibody. Again about 1-2% of untreated cells were antigen positive. Since the KS patient sera contained antibodies to EBV VCA, these antigen-positive cells may represent those cells that spontaneously enter the EBV lytic cycle. Alternatively, they may represent cells that spontaneously produced KSHV lytic cycle antigens. However, n-butyrate caused a 10- to 20-fold increase in the number of BC-1 cells that expressed antigens detectable by KS patient sera. These antigens were cytoplasmic and nuclear. Since n-butyrate treatment had no effect on the number of BC-1 cells expressing EBV lytic cycle antigens, the antigen containing cells detected with KS patient sera were considered to be expressing lytic cycle KSHV polypeptides. Thus, n-butyrate treatment caused 25 to 50% of the BC-1 cell population to switch from latency to the KSHV lytic cycle and produce antigens detectable by immunofluorescence.

These results with the uncloned BC-1 parental line were reproduced in the single cell clones. The number of cells positive for EBV VCA in these clones varied from <1% to 4% and was not altered by n-butyrate treatment. However, n-butyrate treatment induced between 10% and 50% of the cells in each clone to  
5 express lytic cycle KSHV antigens.

Electron microscopy. BC-1 cells were examined by transmission electron microscopy to determine whether n-butyrate induced KSHV to form morphologically recognizable herpesvirions. Two preparations of untreated BC-1 cells, examined over an interval of two months, contained herpes nucleocapsids in  
10 approximately 2.5% of cell profiles (1 of 40 cell profiles examined). However, 48 h following treatment with n-butyrate, 50% to 80% of cell profiles contained herpesvirus nucleocapsids. These were almost exclusively intranuclear and predominantly of the herpesvirus "b" morphologic type. Of 138 nucleocapsids, classified on 15 randomly chosen images, 92% were type "b", 3% were type "a", *i.e.*, lacking  
15 a core, and 5% were type "c" with a dense core (Figure 9). Since n-butyrate had little or no effect on EBV DNA content or EBV late gene expression (Figures 3 to 6) these nucleocapsids are likely to be KSHV. Only a single extracellular particle with the appearance of a morphologically complete herpesvirus particle was detected (Figure 9B). A majority of the n-butyrate treated cells containing herpes-  
20 virus nucleocapsids appeared to have lysed. In addition the infected cells showed extensive nuclear membrane duplication and nuclear formations of electron dense oriented fibrillar material (Figure 9D).

Attempts to demonstrate extracellular KSHV virions. Biochemical and biologic assays were used to assess whether mature KSHV was released from BC-1  
25 cells following induction of the KSHV lytic cycle by n-butyrate. In the biochemical assay an extracellular nucleocapsid preparation was obtained by repeated DNase and NP40 treatment of a high-speed pellet of supernatant fluids from n-butyrate-treated BC-1 cells. These preparations contained EBV DNA (Figure 10, lane 7) but not KSHV DNA in a DNase resistant form (Figure 10, lane 4). KSHV  
30 DNA was present in a DNase resistant form inside cells, suggesting that at least a portion of KSHV DNA was encapsidated.

- 40 -

In biologic experiments supernatant fluids from BC-1 cells that were untreated or treated with chemical inducing agents were added to cultured human umbilical cord lymphocytes (HUCL). When the HUCL were morphologically transformed into lymphoblastoid cell lines, they were shown to contain EBV DNA (Figure 9, panel C, lanes 2-5) but no KSHV DNA (Figure 11, panel B, lanes 2-5). These results, that demonstrated no release of encapsidated or infectious KSHV genomes from BC-1 cells, were consistent with our observations by electron microscopy in which profiles of n-butyrate treated cells, containing large numbers of nuclear and cytoplasmic herpesvirus nucleocapsids, rarely revealed mature enveloped extracellular virions.

## DISCUSSION

These studies allow the following general conclusions about cell-virus relationships in the BC-1 cell line harboring both gammaherpesviruses. The majority, if not all, BC-1 cells contain both genomes. In more than 98% of the cells both viruses are latent; in the other 2% of cells one or both of the viruses spontaneously replicate. Each virus can be independently stimulated into lytic cycle gene expression by the selective action of inducing chemicals, TPA and n-butyrate. Activation of one virus does not activate expression of the other. Both untreated and chemically activated BC-1 cells release infectious EB virus. However, unlike EBV, KSHV DNA is not released from BC-1 cells in an encapsidated form that is resistant to the action of DNase.

Both KSHV and EBV are latent in BC-1 cells. Cell-cloning experiments (Figure 9) suggested that the majority of cells harbor both viruses. However, other recently described cell lines established from body cavity lymphoma have been reported to harbor only KSHV (80). Since EBV is not necessary for outgrowth of cell lines from body-cavity lymphoma, EBV may be a passenger virus in this disease.

Viral latency is spontaneously disrupted in a few cells. Approximately 2% of untreated BC-1 cells react by immunofluorescence with EBV-positive human

sera or with monospecific antisera to EBV late antigens (68, 88). Thus about 2% of BC-1 cells spontaneously enter the EBV lytic cycle. About 2% of cells are also reactive by fluorescence *in situ* hybridization (FISH) with probes for an abundant KSHV-encoded 1.1 kb early RNA. Therefore, a similar number of cells spontaneously enter the KSHV lytic cycle. Electronmicroscopy confirms that about 2% of untreated BC-1 cells make herpesvirions.

Selective chemical activation. TPA markedly activates EBV DNA replication and EBV early and late gene expression in BC-1 cells, while the abundance of KSHV DNA and the late mRNA detected by KS330 *Bam* remains relatively unaffected (Figures 4 to 7). Although TPA preferentially induces EBV lytic cycle genes, it also induces the KSHV-associated p60 protein (Figure 7A). Other studies suggest that p60 is a KSHV early protein whose expression is unaffected by inhibitors of viral DNA synthesis. Furthermore, the abundant KSHV 1.1 kb early RNA can also be induced by TPA in BC-1 cells. These results suggest that in BC-1 cells KSHV early gene expression may be activated by TPA, but n-butyrate is required for KSHV to proceed to lytic viral DNA synthesis and late gene expression. However, in other body cavity lymphoma B cell lines, in which EBV is not present, TPA is sufficient to drive KSHV all the way to virion production (80).

n-butyrate, by itself, strongly activates KSHV gene expression in BC-1 cells, while leaving EBV gene expression relatively unaffected (Figures 4 to 7). Thus, it is unlikely that there exists a KSHV gene product that is able to cross-activate EBV. This finding has permitted the development of first-generation serologic assays using BC-1 cells for detection of antibodies to KSHV lytic cycle antigens in human sera (68). Sera from 70% of HIV-infected patients with KS recognize a marked increase in the abundance of the p40 polypeptide and in the number of antigen-positive BC-1 cells in butyrate-treated cultures. About 12% of HIV-infected patients without KS recognize p40.

Shut-off mechanism accompanying KSHV activation by butyrate. Treatment of BC-1 cell with n-butyrate was associated with a dramatic decrease in the abundance of host cell b actin mRNA (Figure 6). The experiments so far do not permit a distinction between some non-specific "cytotoxic" effect of butyrate or a

KSHV-specific host-cell shut-off mechanism. A cytotoxic effect of butyrate is suggested by the complete disappearance of the cellular b actin signal even though KSHV lytic gene expression and nucleocapsid production was observed in about 50% of the cells. However, several pieces of indirect evidence also favor a specific effect mediated by some KS viral gene product(s). Although n-butyrate treatment of BC-1 cells inhibited cellular b actin and EBV late gene mRNAs, the KSHV late gene mRNAs were unaffected (Figure 6A). Treatment of HH514-16 cells with n-butyrate, causing 50-80% of the cells to enter the EBV lytic cycle, was not accompanied by decreased  $\beta$ -actin mRNA. Furthermore, activation of EBV gene expression by TPA in BC-1 or B95-8 cells was not accompanied by a decrease in cellular b actin mRNA. Thus, butyrate is not toxic to other B lymphoblastoid cells and activation of EBV by itself is not associated with host-cell shut-off. Moreover, TPA activation of KSHV expression in BCBL-1, a body-cavity lymphoma line different from the one studied herein, which does not contain EBV, is also associated with cell lysis (80). These results are consistent with the hypothesis that induction of KSHV lytic gene expression by n-butyrate is associated with a powerful host-cell shut-off mechanism.

The shut-off mechanism may also suppress EBV gene expression. In cells which are treated with both TPA and n-butyrate there is no increase in EBV DNA replication or late lytic mRNA expression, even though TPA by itself strongly activates these processes (Figure 4 and Figure 6). This finding suggests that addition of n-butyrate and its corresponding activation of KSHV expression may shut off EBV lytic cycle gene expression. However, KSHV gene expression is markedly induced in BC-1 cells treated with both chemicals, suggesting that EBV does not possess a reciprocal shut-off mechanism that impairs KSHV expression.

Lack of release of encapsidated KSHV genomes. n-butyrate treatment caused the appearance of numerous intranuclear herpesvirus nucleocapsids in BC-1 cells (Figure 9). Since n-butyrate did not induce any increase in the content of EBV DNA (Figures 4 to 6), EBV late mRNA (Figure 6) or EBV capsid polypeptides (Figure 7) it is likely that most of these viral particles represent KSHV virions. Nonetheless, there appeared to be a defect in the release from BC-1 cells

- 43 -

of particles which contain KSHV genomes in a DNase resistant form. The explanation that BC-1 KSHV nucleocapsids, unlike EBV nucleocapsids, are leaky and permit the entry of DNase seems unlikely since KSHV DNA can be released in a DNase resistant state from BCBL-1, another body cavity based lymphoma cell line (80) and from MH-B2, a recently established cell line. Therefore B cells can be competent to release encapsidated KSHV. Moreover, intracellular nucleocapsids from n-butyrate treated BC-1 cells contain KSHV DNA in a DNase resistant form. Another explanation is that there is some defect in KSHV maturation in BC-1 cells. Only a single extracellular virion was observed by electron microscopy of BC-1 cells; this is likely to be EBV. It is not yet known whether the KSHV genome in BC-1 cells contains all of the KSHV genes in an intact form or represents a partially defective genome, such as are regularly found in lymphoid tumor cells transformed by *Herpesvirus saimiri* (23,31) and frequently encountered in several EBV lineages as well (52). BC-1 cells may release infectious KSHV that was not detectable by the biochemical or biologic assays we used. Moore et al. have reported that co-cultivation of BC-1 cells with Raji cells in communicating chambers separated by a 0.45m membrane filter, resulted in the transfer of KSHV genome to Raji cells (70). These experiments suggest that infectious KSHV may be released from BC-1 cells. Alternatively, unencapsidated KSHV DNA may have been transferred from BC-1 to Raji cells. The resolution of this problem awaits the development of sensitive infectivity assays for KSHV.

Elements of the latency to lytic cycle switch of the two gammaherpesviruses in BC-1 cells. There is a classical pattern of EBV gene expression in BC-1 cells. The cells express EBNA1 and other latency products that have not yet been characterized. Treatment with TPA is accompanied within 6 hours by increased expression of BZLF1 mRNA and ZEBRA protein. Presumably ZEBRA drives the lytic cascade. The components of the switch in KSHV are not yet characterized. It is not yet known which KSHV gene products are expressed during latency. KSHV is not known to have a homologue to EBV BZLF1; its closest relative *Herpesvirus saimiri* does not have such a homologue (1). However all three gamma herpesviruses do possess variants of the EBV BRLF1 transactivator (1). The R transacti-

vator, perhaps in combination with other immediate early genes, is likely to control the latency to lytic cycle switch in KSHV.

A striking biologic observation in the BC-1 cell system was the independent inducibility of the two viruses in the same cell. Previously, it has been found that in some cell backgrounds, *e.g.*, B95-8 marmoset cells, EBV is preferentially induced by TPA and unaffected by butyrate; in other cell backgrounds, *e.g.*, HH514-16, EBV is preferentially induced by *n*-butyrate and unaltered by TPA. However since these different species of cells also harbor different EBV strains, it was not known whether differences in inducibility were a result of viral or cell differences or both. The current observations of different chemical inducibility of two gammaherpes viruses in the same clonal cell background (*e.g.*, see Figure 6A and 6B and Figure 8B) point to the role of some viral component in the determination of response to chemical activation of the lytic cycle.

### Example 3

#### 15 Identification, Expression and Immunogenicity of Kaposi's Sarcoma Associated Herpesvirus Encoded Small Viral Capsid Antigen

Cell culture. BC-1 cells (16) were grown in RPMI 1640 supplemented with 15% fetal bovine serum at 37°C in the presence of 5% CO<sub>2</sub>. HH514-16 cells derived from the African Burkitt's lymphoma line, P3J-HR-1, were grown in RPMI 1640 supplemented with 8% fetal bovine serum at 37°C (78). Both EBV and KSHV genomes are present in BC-1 cells while only EBV DNA is present in HH514-16 cells. To induce lytic cycle gene expression cells were harvested after exposure to 3 mM sodium butyrate and/or 20 ng/ml TPA (phorbol 12-myristate 13-acetate). COS-7 and 293T cells were used for mammalian cell expression (40, 74).

25 cDNA library construction and cloning. Total cellular RNA was extracted by standard procedures (6) from BC-1 cells which had been treated with sodium butyrate for 48 hr. The poly(A)-plus RNA was selected on an oligo dT column. cDNAs were synthesized using a commercail kit (ZAP cDNA Synthesis Kit,

- 45 -

Stratagene). The library was cloned in the LambdaZAPII vector (Stratagene, CA) according to the manufacturer's protocol. Isolated clones were plaque purified before *in vivo* excision.

5        Genomic library. Total genomic DNA prepared from BC-1 cells by standard procedures (6) was partially digested with Sau3A so that the DNA fragments were 20-40 kb in size. DNA was then ligated to a cosmid vector Supercos-1 (Stratagene, CA) or to a lambda phage vector lFix (Stratagene, CA) and packaged with gigapack LX (Stratagene, CA).

10       DNA sequence analysis. The sequences of cDNA clone CA20 and its gene were determined in both directions via primer walking. DNA sequence data were compiled and analyzed using GELASSEMBLE, TESTCODE, BLAST, FRAMES of Wisconsin Sequence Analysis Package GCG, version 8. (Genetics Computer Group, Madison, Wisconsin).

15       RNA preparation and Northern blot analysis. Each sample contained total RNA prepared from  $2 \times 10^6$  cells; RNA was fractionated on 1% formaldehyde agarose gels and transferred to a nylon membrane (Nytran, Schleicher & Schuell) by standard procedures (6). The DNA probe for Northern blotting was a 620-bp PCR product of CA20, amplified by primers 17809 (5'-tggaccatggccaactttaaggtgagacc-3', SEQ ID NO. 6) and 17810 (5'-ggaattcaacaaaagtggccgcctatcgg-3',  
20       SEQ ID NO. 7). Hybridization was carried out in 50% formaldehyde, 5x SSC, 5x Denhardt's solution, 1% SDS, and 100µg/ml salmon sperm DNA at 45°C overnight. Filters were washed with 3x SSC, 0.1% SDS twice for 15 min each and followed by 0.1 x SSC, 0.1% SDS once for 30 min at 68°C. For quantitation of RNA loading, blots were stripped and reprobbed with genes for RNase P H1 RNA (7).  
25       Band intensity was quantitated with a PhosphorImager (Molecular Dynamics).

30       Expression of KSHV sVCA in mammalian cells. Ten µg of each plasmid DNA was transfected into 75 % confluent COS-7 or 293T cells by the calcium phosphate precipitation method for 5 hr followed by a 10% glycerol shock for 2 min at the end of transfection (6). Three days after transfection, total cellular proteins were prepared for Western blotting.



In Vitro translation and immunoprecipitation. A commercial *in vitro* transcription and translation system, TNT<sup>®</sup> Coupled Reticulocyte Lysate System (Promega, WI), was employed to generate the *in vitro* translation product of KSHV sVCA. One µg of plasmid DNA containing the CA20 cDNA downstream of a T3 promoter in pBK-CMV (Stratagene, CA) was added to 50 ml of TNT<sup>®</sup> reaction in the presence of [<sup>35</sup>S]-methionine of 800 mCi/ml. Reactions were incubated at 30°C for 90 min. Translation products were analyzed on 12% SDS-PAGE and visualized by autoradiography or directly used in immunoprecipitation. For immunoprecipitation, 2 ml of TNT<sup>®</sup> product, 5 ml serum, and 1.5 mg of protein A-sepharose CL-4B (Pharmacia, NJ) were added to 400 ml radio-immunoprecipitation/bovine serum albumin (RIP/BSA) buffer (10mM Tris-HCl[pH8.0], 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 100mM NaCl, 1% BSA) and incubated at 4°C for 2 hr. Protein A-sepharose beads were washed thoroughly in 1x RIP buffer three times followed by one washing in 10mM Tris-HCl [pH 6.8]. The immunoprecipitation complex was resolved on 12% SDS-PAGE and detected by autoradiography.

Expression of KSHV sVCA in *Escherichia coli* and partial purification.

The open reading frame of KSHV sVCA was amplified by PCR from CA20 using primers 17809 (5'-tggaccatggccaactttaaggtgagagacc-3', SEQ ID NO. 6) and 17810 (5'-ggaattcaacaaaaagtggccgcctatcgg-3', SEQ ID NO. 7). The resulting PCR product was subcloned into the pET30b vector (Novagen, WI) using *NcoI* and *EcoRI* sites (underlined), yielding the recombinant plasmid pET30b-sVCA. *E.coli* BL21(DE3)pLysS harboring pET30b-sVCA in logarithmic phase growth was induced by 0.1mM IPTG for 2 hr at 37°C. A crude extract of the induced cells was prepared by 1:20 concentration of the cell pellet in 50mM Tris-HCl (pH8.0). Purification of KSHV sVCA was carried out by chromatography of crude extracts onto two consecutive affinity columns, a Nickel column and S•Tag<sup>™</sup> agarose (Novagen, WI). The elution buffer used in the Nickel column was a gradient of 0.1-1.0 M imidazole in 50 mM NaCl, 20 mM Tris-HCl (pH 8.0). The elution buffer used for S•Tag<sup>™</sup> agarose was 0.5% SDS in 20mM Tris-HCl(pH 7.5), 0.15 M NaCl, and 0.1 % Triton X-100.

Immunization of rabbits. Nickel column eluents containing the sVCA protein were electrophoresed on an 10% preparative SDS-polyacrylamide gel. The gel was stained with Coomassie brilliant blue and strips containing the sVCA were ground and emulsified in complete Freund adjuvant (0.5 ml per rabbit). Rabbits  
5 were immunized by subcutaneous injection and boosted every two weeks with an equivalent amount of protein emulsified in incomplete Freund adjuvant (0.5 ml per rabbit). Serum was collected 1 to 2 weeks following each boost.

Western immunoblot analysis. After SDS-PAGE, proteins were transferred to nitrocellulose filters (Schleicher & Schuell) by standard procedures (6). The  
10 filters were incubated with blocking buffer (5% non-fat dried milk, 10mM Tris-HCl (pH 7.4, 30 mM NaCl) for 1 hr at room temperature. A 1:150 dilution of serum was added for 2 hr at room temperature or overnight at 4°C. Filters were subsequently washed in Tween 20-Tris buffer (0.2% Tween 20, 0.15M NaCl, 10 mM Tris-HCl (pH7.0) three times for 10 min each. After washing the filters in Tween  
15 20-Tris buffer, (<sup>125</sup>I) labeled protein A was added at 1:2000 dilution to trace the bands by autoradiography. The Cassette Miniblot System (Immunitics, MA) was used to screen 25 sera at one time. One and one half µl of serum diluted 1:33 in blocking buffer was used in each channel of the miniblot apparatus.

Sera. Sera were collected from HIV-infected patients in Connecticut, New  
20 York and California (68). Other sera from patients with hemophilia, autoimmune disease, febrile illness, nasopharyngeal carcinoma, children born to HIV-seropositive mothers or healthy subjects were from serum collections.

Nucleotide sequences accession number. The cDNA sequence reported herein has been deposited into Genbank under accession number U50141.

25

## RESULTS

Molecular cloning of KSHV sVCA. Sera from KS patients recognize KSHV associated polypeptides in butyrate-treated BC-1 cells. These polypeptides were assumed to represent the lytic cycle gene products of KSHV (65, 68). To clone lytic cycle antigens about 10<sup>6</sup> plaques of a cDNA library constructed from

BC-1 cells induced by Na-butyrate for 48 hr were immunoscreened with KS patient serum 0103. Positive phage clones were identified and subjected to *in vivo* excision. A partial nucleotide sequence of the insert in each plasmid was determined from both ends; homologs were sought in the data base. Among 54 clones analyzed, 11 clones showed significant homology to gammaherpesviral open reading frames (ORF) which encode small viral capsid antigen components, namely EBV BFRF3 and HVS ORF65 (1, 105). After alignment of these 11 clones, clone CA20 was chosen for further study since it was likely to represent a full length cDNA. The genomic counterpart of CA20 was obtained from cosmids which contained other KSHV sequences including KS631Bam (17). DNA sequences of this 716 bp-long cDNA as well as its gene are summarized in Figure 12. The putative TATA box (TATTAAA) was located 50 bp upstream of the start of CA20 cDNA; the polyadenylation signal (AATAAA) was located 22 bp upstream of the polyadenylation site. The ORF of CA20 would encode a 170 aa polypeptide which was designated KSHV small viral capsid antigen (sVCA) (Figure 13). Amino acid sequences of KSHV sVCA shared 48% similarity and 27% identity to EBV BFRF3 and 60% similarity and 40% identity to HVS ORF65, suggesting these three ORFs may possess related biological functions (Figure 13).

Detection of KSHV sVCA transcripts in BC-1 cells. To study the kinetics of expression of KSHV sVCA, a PCR product representing the coding region of CA20 was used as a probe to detect mRNAs prepared from chemically treated BC-1. As shown in Figure 14, expression of KSHV sVCA was detected after chemical treatment of KSHV-positive cells (lanes 5-7, 13-15), but not of KSHV-negative EBV-positive HH514-16 cells (lanes 2 and 10). The size of the major transcript, 0.85 kb, was consistent with the size of the cDNA clone which was 716 nt. This stable transcript was detectable between 12 and 18 hr after chemical induction; its expression was sustained for at least 48 hr (Figure 14). Na-butyrate induced a higher level of expression of the 0.85 kb sVCA mRNA than did TPA, as previously shown for the mRNA detected by KS330Bam, which represents the gene for another capsid component (65).

KSHV sVCA is encoded by a viral lytic late gene. To determine the stage of the viral life cycle in which KSHV sVCA was expressed, BC-1 cells were pre-treated with a protein synthesis inhibitor, cycloheximide, or with herpesvirus DNA polymerase inhibitors, phosphonoacetic acid (PAA), phosphonoformic acid (PFA), or acycloguanosine (ACG) before addition of butyrate as the inducing agent. Expression of the 0.85 kb sVCA mRNA was dramatically inhibited by addition of either cycloheximide, PAA, or PFA (Figure 14). Since protein synthesis and viral DNA replication were required for its expression, KSHV sVCA could be classified as a late gene product. sVCA expression was not inhibited by ACG. It is not known whether ACG resistance resulted from insensitivity of the BC-1 KSHV thymidine kinase or DNA polymerase (34, 73, 103).

Expression of KSHV sVCA in mammalian cells. sVCA was transiently expressed in COS-7 or 293T cells transfected with pBK-CMV vector containing the CA20 cDNA. A protein band estimated at 30 kDa was recognized by KS patient sera on immunoblots prepared from extracts of both cell types (Figure 15). The 30 kDa protein represents a fusion between 56 aa of b-galactosidase encoded in the vector to the 170 aa of the sVCA ORF. These results suggested that CA20 encoded an antigen recognized by KS sera.

In vitro translation of KSHV sVCA. To determine the intrinsic molecular weight of KSHV sVCA ORF, the protein was synthesized in a rabbit reticulocyte lysate (Figure 15B). In this experimental system the sVCA protein was not fused to another peptide; the first ATG of KSHV sVCA was used as the initiation codon. A predominant 22 kDa protein was seen when plasmid DNA containing the sVCA gene served as a template for *in vitro* transcription and translation (Figure 15B, lane 2). Like the sVCA encoded by BFRF3 of EBV the migration was slightly slower than predicted (88). The size of *in vitro* translated sVCA corresponded to that expressed in chemically treated BC-1 cells (Figure 17D). The 22 kDa *in vitro* translated product was immunoprecipitated by sera from patients with KS (lanes 3-6) but not by control sera (lanes 7-10). These results provided further evidence that KSHV sVCA was a potential serological marker for KS.

Expression of KSHV sVCA in *Escherichia coli*. To obtain large quantities of protein for further serological studies, sVCA ORF was expressed in *E. coli* using the pET30b system (Novagen). A 44-aa peptide containing His•Tag<sup>®</sup> and S•Tag<sup>™</sup> encoded in the vector was fused to the N terminus of sVCA. A protein band with an estimated molecular weight of 29 kDa was detected by immunoblotting in extracts from *E. coli* harboring pET30b-sVCA (Figure 15C, lane 3), but not in extracts of control cells containing vector alone (lane 1) or plasmid containing an unrelated insert (lane 2). These results suggested that immunoreactive epitopes of KSHV sVCA were retained when expressed in *E. coli*.

KSHV sVCA was detected as a 22 kDa viral lytic protein in BC-1 cells. Antisera to sVCA were raised in rabbits immunized with the protein expressed in *E. coli*. These antisera recognized a 22 kDa protein in BC-1 cells treated with Na-butyrate (Figure 15D, lane 2) or TPA plus Na-butyrate (lane 4), but only faintly detected the protein in cells treated with TPA (lane 3). The size of the polypeptide corresponded to the *in vitro* translation product (Figure 15B); its pattern of expression following chemical induction corresponded to that of the sVCA 0.85 kb mRNA (Figure 14A). Therefore, the 22 kDa immunoreactive protein in BC-1 cells is KSHV sVCA.

KSHV sVCA does not share cross-immunogenicity with EBV BFRF3. EBV infection is widespread in the general population; antibodies to EBV BFRF3 represent an immunodominant serological marker for the past EBV infection (89, 104). EBV BFRF3 and KSHV sVCA are 48% similar at the level of primary amino acid sequence. If KSHV sVCA was to be used as a serological marker, it was essential to determine whether any cross-reactivity existed between these two related proteins. Therefore, 10 EBV positive sera were tested for antibodies to KSHV sVCA using Western immunoblot analysis (Figure 16). Protein extracts prepared from *E. coli* containing either pET30b alone or recombinant pET30b-sVCA were run in parallel in each test. The five sera from KS patients reacted with KSHV sVCA; none of the other EBV positive sera recognized the 29 kDa sVCA fusion protein. In an independent experiment, a healthy donor serum which has been used as a reference positive control for antibodies to EBV BFRF3 failed

to detect KSHV sVCA. Moreover, rabbit antibodies to KSHV sVCA did not react with BFRF3 expressed in mammalian cells. These results indicate that EBV BFRF3 and KSHV sVCA, although similar at the level of primary amino acid sequence, do not present cross-reactive epitopes in the milieu of the human immune system.

5                    Purification of KSHV sVCA. With the aid of two tags at its N-terminus, His•Tag® and S•Tag™, KSHV sVCA was partially purified from *E.coli* extracts using two consecutive affinity columns (Figure 17). A highly purified soluble form of KSHV sVCA was present in fractions 3 to 7 eluted from the S•Tag™ agarose  
10 affinity column. The antigenicity of the purified protein was preserved on immunoblot analysis and provided material for large scale screening (Figure 18).

Prevalence of antibodies to KSHV sVCA. There was a high correlation between the presence of antibody to p40 antigen in butyrate-treated KSHV-infected cells and presence of antibody to sVCA in HIV positive patients. 31 of 34 (91%)  
15 sera with antibody to p40 also contained antibody to sVCA. However, among HIV-infected patients antibody to sVCA was detected in 23 of 67 sera lacking antibody to p40. Thus antibody to sVCA appeared to be a more sensitive serological marker than antibody to p40. Nearly 90% of HIV-infected patients with KS were seropositive (Table 6). In other studies it was found that more than 90% of  
20 patients with Mediterranean KS and post-transplant KS also react to sVCA. 20% of HIV-infected patients without KS were seropositive to sVCA. Antibodies to sVCA were not detected in patients from the USA with hemophilia, autoimmune disease, or acute viral illness. Nor were antibodies detected in children born to  
25 HIV-seropositive mothers. Antibodies were not present in sera from Chinese patients with nasopharyngeal carcinoma. Since these sera have extremely high antibody titers to EBV proteins, the results underscore the lack of immunologic cross-reactivity between sVCA and EBV proteins. Three sera from 28 healthy laboratory workers showed weak seroreactivity.

**Table 6.** Prevalence of Antibody to KSHV sVCA in Various Populations

	<u>Sample Profile</u>	<u>Source</u>	<u>Ab to KSHV sVCA</u>
	HIV-positive with KS	USA	42/47 (89%)
	HIV-positive without KS	USA	11/54 (20%)
5	HIV-positive children	USA	0/12
	HIV-negative children	USA	0/10
	Hemophilia patients	USA	0/25
	Autoimmune patients	USA	0/25
	Children with acute illness	USA	0/25
10	Healthy Adults	USA	3/28 (11%)
	Nasopharyngeal Cancer China		0/25

## DISCUSSION

15 A KSHV encoded protein with homology to small virion capsid components  
 of other herpesviruses is identified and cloned in this Example. This small viral  
 capsid component is immunogenic in man and can serve as a serological marker for  
 infection with Kaposi's sarcoma associated herpesvirus. KSHV sVCA is homolo-  
 gous to structural components of several other herpesviruses including VP26 of  
 HSV, BFRF3 of EBV, ORF65 of HVS and the recently identified smallest capsid  
 20 protein (SCP) of human cytomegalovirus (38). Despite this homology KSHV  
 sVCA does not appear to be antigenically crossreactive. KSHV sVCA is similar in  
 size to its counterparts among the gammaherpesvirus (Figure 12B); however the  
 comparable proteins among alpha- and betaherpesvirus are smaller, in the range of  
 10-12 kDa (21, 61). All proteins of this group are highly basic. KSHV sVCA  
 25 contains 12% arginine and lysine and has a predicted pI of 10.45. Among the  
 gammaherpesvirus, the homology extends throughout the proteins (Figure 12B).  
 The smaller HSV VP26 and cytomegalovirus SCP are homologous to the C-  
 terminal portion of KSHV of sVCA.

Among this group of related herpesvirus structural components only HSV VP26 has been studied in any detail (11, 102, 108). Computer analysis of images collected by cryoelectron microscopy show that each hexon tip is surrounded by six copies of VP26 (11, 102); however, VP26 does not associate with the penton (102).  
5 The *in vitro* assembly by HSV nucleocapsids from components purified from a baculovirus expression system does not require VP26 (100). A comparison of capsid structures with or without VP26 suggests that the small virion protein may play a role in linking the viral capsid to outer virion structures (11). The location of this group of proteins on the outer surface of the capsid may contribute to their  
10 immunodominant behavior.

This is the first report in which a recombinant viral gene product was used as an antigen to determine the prevalence of antibodies to KSHV in different populations. A high seroreactivity rate against KSHV sVCA among patients with different types of KS: 89 % among AIDS KS, 93 % among post-transplant KS and  
15 95 % among classic KS (Table 6) was observed. This rate of seroreactivity among KS patients is higher than in that previously reported for antibodies to KSHV antigens present in B cells (36, 37, 48, 68). The frequency of antibodies to inducible p40 (68), latent nuclear antigens p226/234 (36, 37) and latency-associated  
20 nuclear antigen (48) detectable by immunofluorescence ranged from 67% to 83% of patients with KS. The recombinant sVCA antigen may therefore be a more sensitive serological marker than antigens present in infected cells. It was found that 20% of sera from HIV-infected patients without KS contained antibodies to KSHV sVCA. This frequency is similar to that of other studies using antigens prepared from KSHV infected cell lines. In those studies 13% to 30% of HIV-  
25 infected homosexual patients were seropositive. Also in agreement with three other studies which used cell antigens a high frequency of antibodies to KSHV sVCA in patients with hemophilia, acute viral syndromes, autoimmune disease or healthy donors (36, 48) was not detected.

The low frequency of antibodies to KSHV sVCA in the general population  
30 stands in marked contrast to the pattern of antibodies to its EBV homologue, BFRF3. Antibodies to BFRF3 are present in nearly all human adults since EBV



infection is nearly universal in man. This comparison of seroepidemiology between EBV and KSHV suggests two alternate possibilities: i) KSHV infection, unlike EBV infection, is not ubiquitous in the human population or ii) antibodies to KSHV sVCA, unlike those against EBV sVCA, do not reflect KSHV infection.

5

The above description is for the purpose of teaching the person of ordinary skill in the art how to practice the present invention, and it is not intended to detail all those obvious modifications and variations of it which will become apparent to the skilled worker upon reading the description. It is intended, however, that all such obvious modifications and variations be included within the scope of the present invention, which is defined by the following claims. The claims are intended to cover the claimed components and steps in any sequence which is effective to meet the objectives there intended, unless the context specifically indicates the contrary.

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The cited papers listed above are hereby incorporated herein in their entireties by reference.

SEQUENCE LISTING

## (1) GENERAL INFORMATION

- (i) APPLICANTS: George Miller  
Michael Rigsby  
Lee Heston  
Elizabeth Grogan  
Ren Sun  
Su-Fang Lin
- (ii) TITLE OF INVENTION:
- (iii) NUMBER OF SEQUENCES: 7
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- (ix) TELECOMMUNICATION INFORMATION
  - (A) TELEPHONE NUMBER: 203-324-6155
  - (B) TELEFAX NUMBER: 203-327-1096

- 66 -

## (2) INFORMATION FOR SEQ ID NO: 1

## (i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 836 residues
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE

- (A) DESCRIPTION: cDNA

## (v) FRAGMENT TYPE: entire sequence

## (vi) IMMEDIATE SOURCE:

## (ix) FEATURE

- (A) NAME: small viral capsid antigen

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO 1:

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TGAGGCTATT TCGCCCGCCT GTGTGGAAGA TGTCCAACCTT TAAGGTGAGA  200
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TGGACAACCG TGGAGTCACA AGTACCACTT CTTTATTCTG TCAACCGTCT   750
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AAACTAATCA GTGTTATCAA AAAAAAAAAA AAAAAA                        836

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- 67 -

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## (i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 34 residues
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- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE

- (A) DESCRIPTION: KSHV primer used in constructs

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO 2:

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## (4) INFORMATION FOR SEQ ID NO: 3

## (i) SEQUENCE CHARACTERISTICS

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- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE

- (A) DESCRIPTION: KSHV primer used in constructs

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO 3:

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## (5) INFORMATION FOR SEQ ID NO: 4

## (i) SEQUENCE CHARACTERISTICS

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- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE

- (A) DESCRIPTION: EBV primer used in constructs

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO 4:

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## (6) INFORMATION FOR SEQ ID NO: 5

- 68 -

- (i) SEQUENCE CHARACTERISTICS
  - (A) LENGTH: 20 residues
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE
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- (xi) SEQUENCE DESCRIPTION: SEQ ID NO 5:  
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- (7) INFORMATION FOR SEQ ID NO: 6
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    - (B) TYPE: nucleic acid
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    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE
    - (A) DESCRIPTION: 17809 primer used in constructs
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO 6:  
TGGACCATGG CCAACTTTAA GGTGAGAGAC C 31

- (8) INFORMATION FOR SEQ ID NO: 7
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    - (B) TYPE: nucleic acid
    - (C) STRANDEDNESS: single
    - (D) TOPOLOGY: linear
  - (ii) MOLECULE TYPE
    - (A) DESCRIPTION: 17810 primer used in constructs
  - (xi) SEQUENCE DESCRIPTION: SEQ ID NO 7:  
GGAATTCAACA AAAAGTGGC CGCCCTATCG G 31

### Claims

1. A method of diagnosing a DNA virus associated with Kaposi's sarcoma which comprises (a) obtaining a suitable bodily fluid sample from a subject, (b) contacting the suitable bodily fluid of the subject to a support having already bound thereto a Kaposi's sarcoma antibody, so as to bind Kaposi's sarcoma antibody to a specific Kaposi's sarcoma antigen, (c) removing unbound bodily fluid from the support, and (d) determining the level of Kaposi's sarcoma antibody bound by the Kaposi's sarcoma antigen, thereby diagnosing Kaposi's sarcoma.
2. A method of diagnosing a DNA virus associated with Kaposi's sarcoma which comprises (a) obtaining a suitable bodily fluid sample from a subject, (b) contacting the suitable bodily fluid of the subject to a support having already bound thereto a Kaposi's sarcoma antigen, so as to bind Kaposi's sarcoma antigen to a specific Kaposi's sarcoma antibody, (c) removing unbound bodily fluid from the support, and (d) determining the level of Kaposi's sarcoma antigen bound by the Kaposi's sarcoma antibody, thereby diagnosing Kaposi's sarcoma.
3. A method according to claims 1 or 2 wherein the DNA virus is human Kaposi's sarcoma-associated herpesvirus, and the antigen is a viral lytic cycle antigen.
4. A method according to claims 1 or 2 wherein the antigen is Kaposi's sarcoma-associated herpesvirus small viral capsid antigen.
5. A method for screening for the presence or absence of antibodies to Kaposi's sarcoma-associated herpesvirus in a patient comprising obtaining a biological sample from the patient and assaying for antibodies to Kaposi's sarcoma-associated herpesvirus small viral capsid antigen or other lytic cycle antigen in the sample.



- 70 -

sample from the patient and assaying for antibodies to Kaposi's sarcoma-associated herpesvirus small viral capsid antigen or other lytic cycle antigen in the sample.

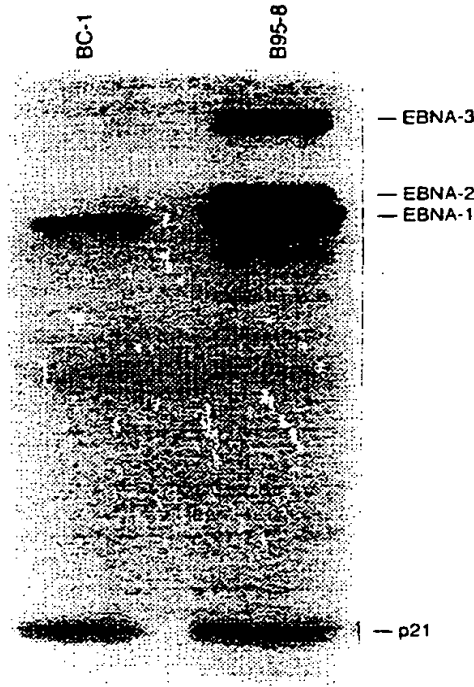
6. A method according to claim 5 wherein the sample is a bodily fluid.
7. A method according to claim 6 wherein the sample is serum.
8. A method according to claim 4 wherein the antigen is encoded by the DNA sequence set out in SEQ ID NO 1, or a sequence that will hybridize to the sequence of SEQ ID NO 1.
9. A method according to claim 5 wherein the estimated level of antibodies to lytic cycle antigen in the sample is determined and compared with a control sample containing a known amount of antibody.
10. A method according to claim 5 wherein the estimated level of antibodies to lytic cycle antigen in the sample is further compared with a second control containing no antibody.
11. A method according to claim 5 wherein the assay for antibody is selected from the group consisting of immunoblotting, immunoassay, and immunofluorescence.
12. A method for screening for the presence or absence of Kaposi's sarcoma-associated herpesvirus antigen in a patient comprising obtaining a biological sample from the patient and assaying for Kaposi's sarcoma-associated herpesvirus small viral capsid antigen or other lytic cycle antigen in the sample.
13. A method according to claim 12 wherein the sample is a bodily fluid.

14. A method according to claim 12 wherein the antigen is encoded by the DNA sequence set out in SEQ ID NO 1, or a sequence that will hybridize to the sequence of SEQ ID NO 1.
15. A method according to claim 12 wherein the estimated level of antigen in the sample is determined and compared with a control sample containing a known amount of antigen.
16. A method according to claim 12 wherein the estimated level of antigen is further compared to a second control containing no antigen.
17. An isolated nucleic acid molecule comprising a sequence selected from the group consisting of:
- (a) a sequence of a genomic DNA clone or a cDNA encoding Kaposi's sarcoma-associated herpesvirus small viral capsid antigen, wherein the noncoding strand of said DNA or cDNA hybridizes under stringent conditions with a DNA probe having the sequence shown in SEQ ID NO 1;
  - (b) a sequence degenerate with the sequence of (a); and
  - (c) a sequence complementary to the full length of the nucleic acid of (a) or (b).
18. A nucleic acid molecule according to claim 17 which is DNA.
19. A nucleic acid molecule according to claim 17 which is RNA.
20. A nucleic acid molecule according to claim 17 which encodes human Kaposi's sarcoma-associated herpesvirus virus small viral capsid antigen.
21. An expression vector comprising a sequence according to claim 17.

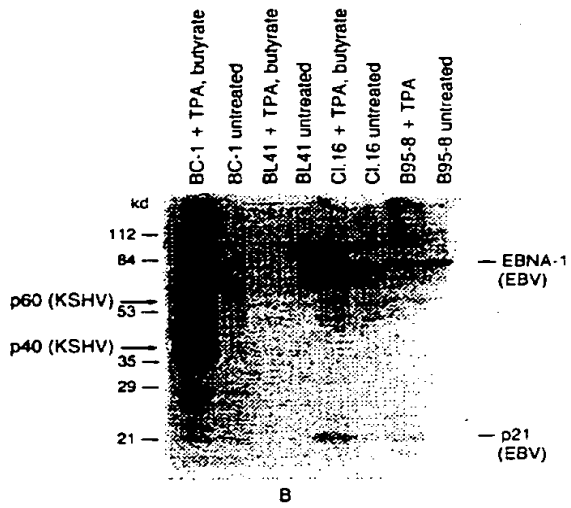
- 72 -

22. A host cell transformed or transfected with a nucleic acid according to claim 17.
23. A polypeptide encoded by a nucleic acid of claim 17.
24. An immunologic assay for Kaposi's sarcoma employing a polypeptide according to claim 17.
25. An isolated DNA molecule comprising the sequence shown in SEQ ID NO 1.
26. An isolated DNA molecule according to claim 25 comprising residues 180 to 689 of SEQ ID NO 1.
27. A polypeptide encoded by the sequence of claim 26.
28. A vector or host cell comprising DNA having the sequence of a DNA molecule according to claim 25.

1/18



A



B

Figure 1

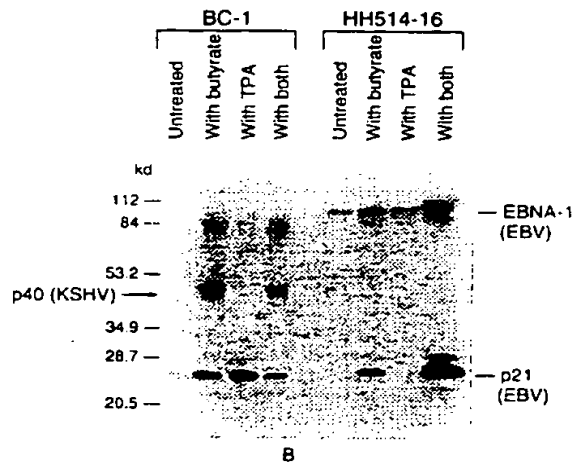
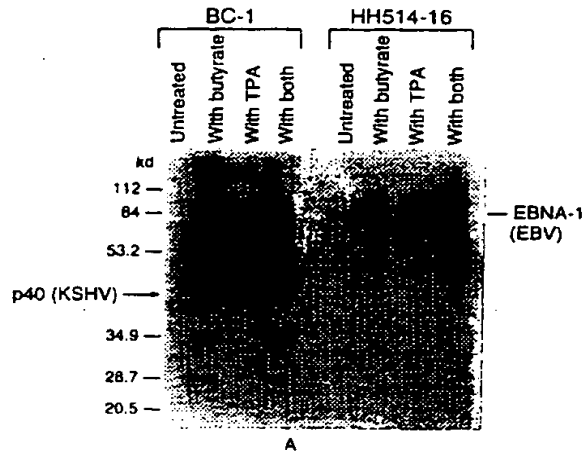
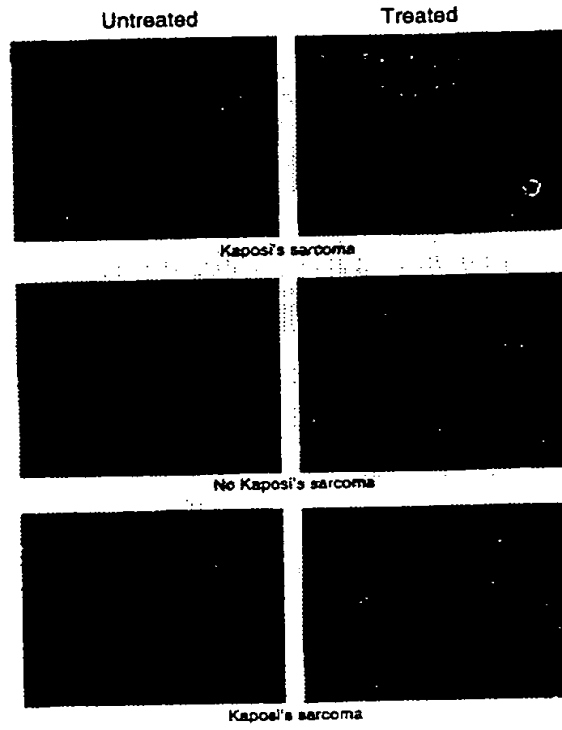


Figure 2

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3/18



**Figure 3**

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316 MILLER ET AL.

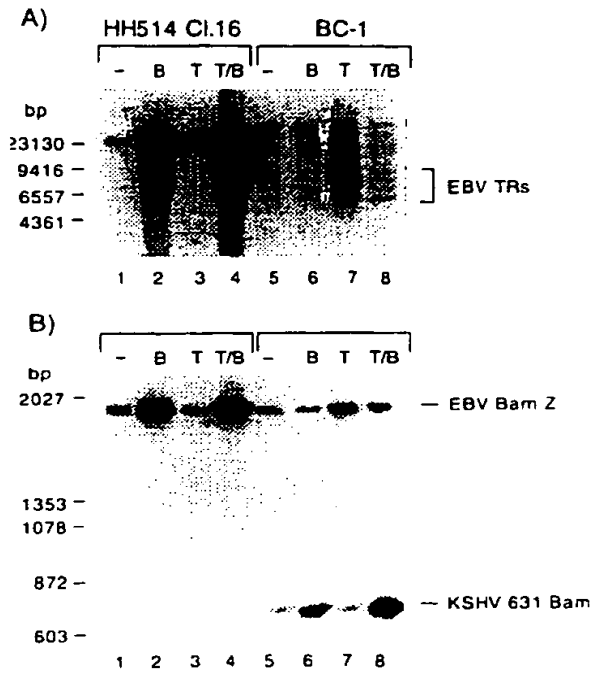


Figure 4

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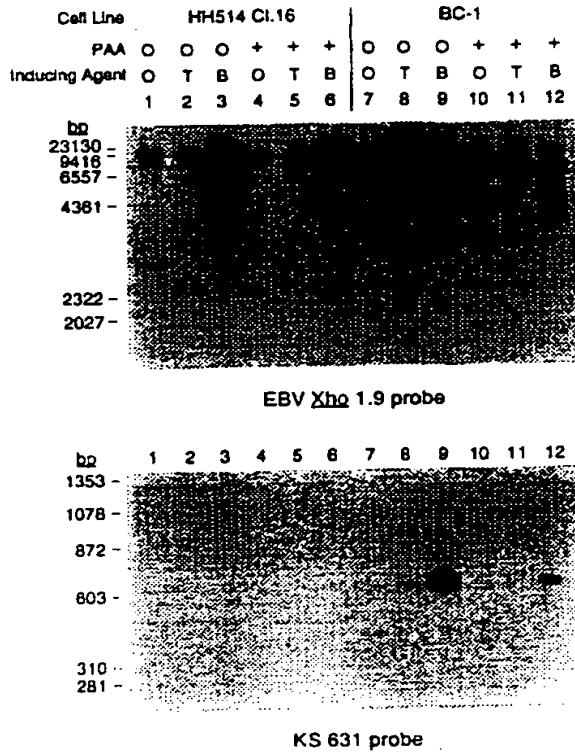


Figure 5

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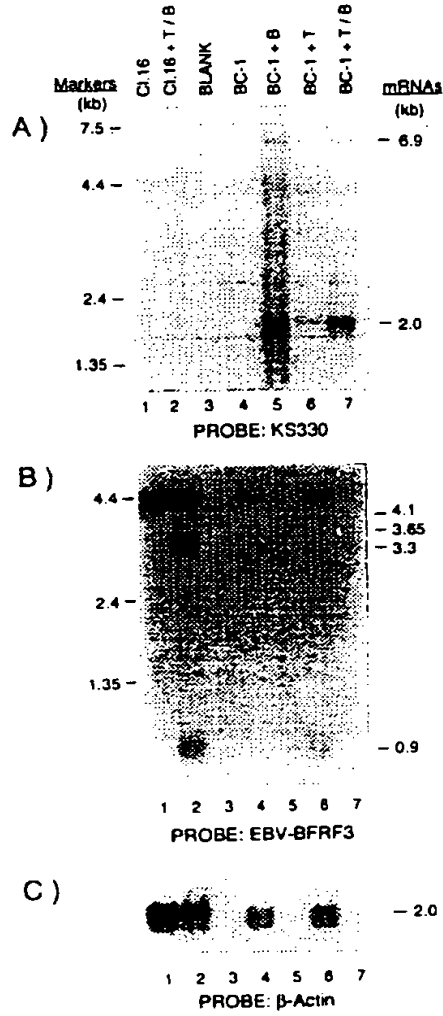


Figure 6

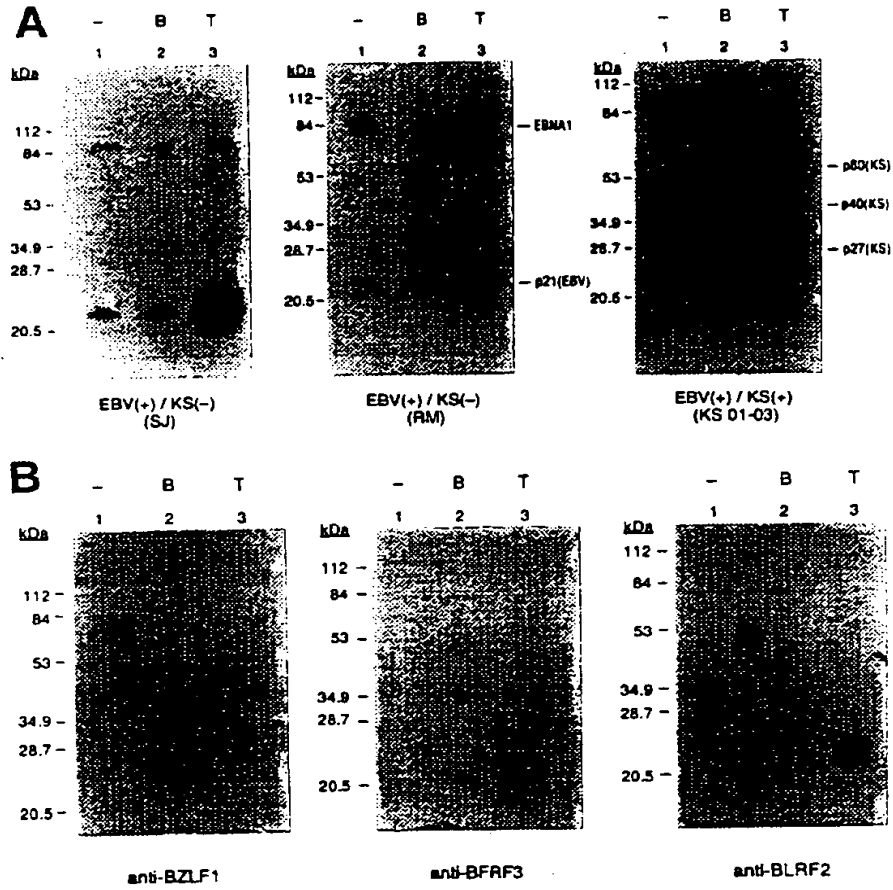


Figure 7

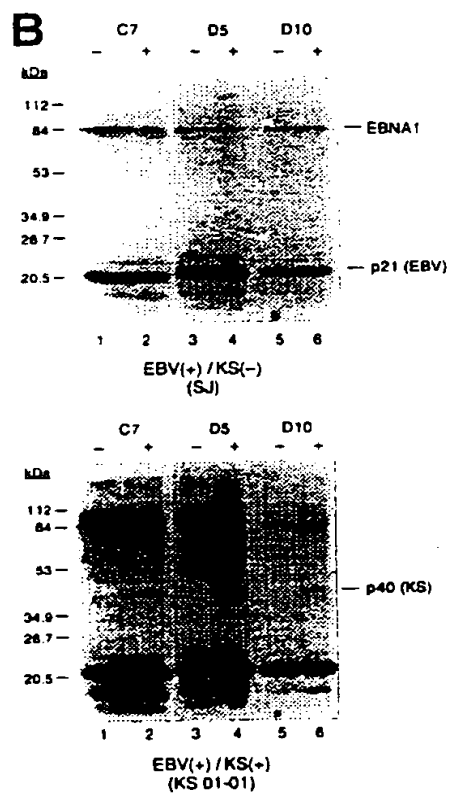
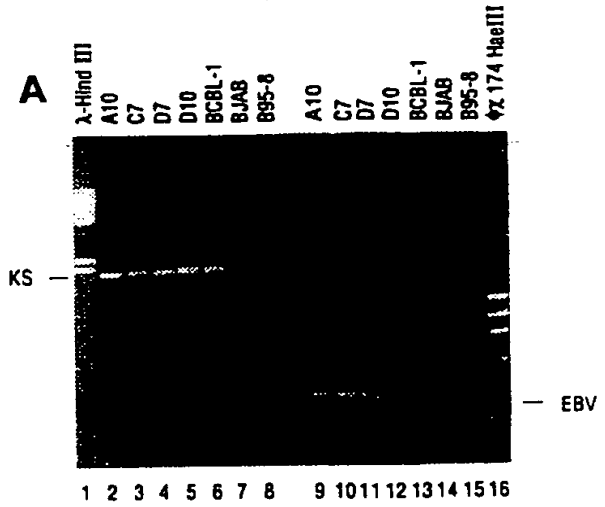


Figure 8

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**Figure 9**  
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10/18

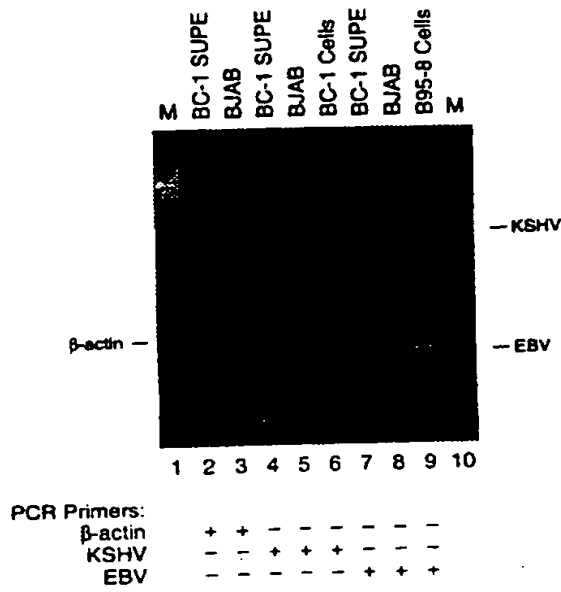


Figure 10

11/18

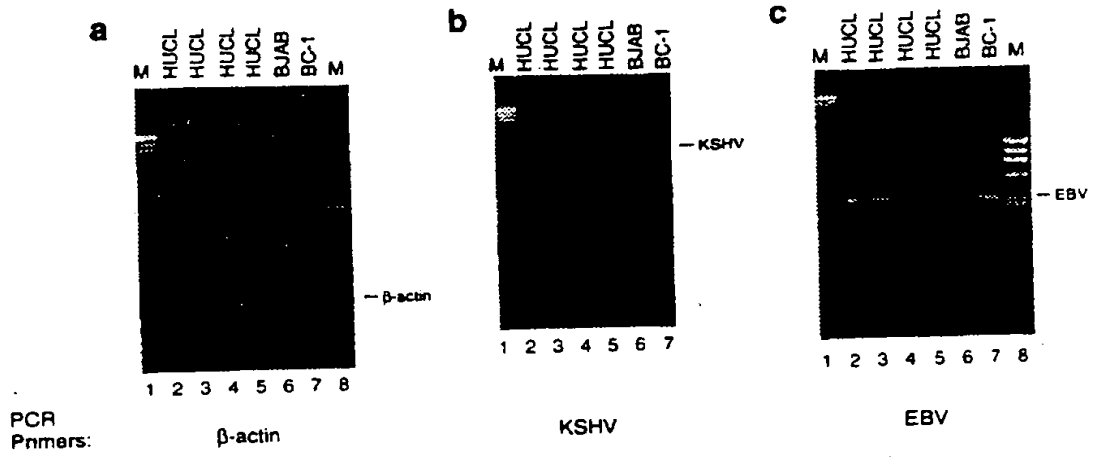


Figure 11

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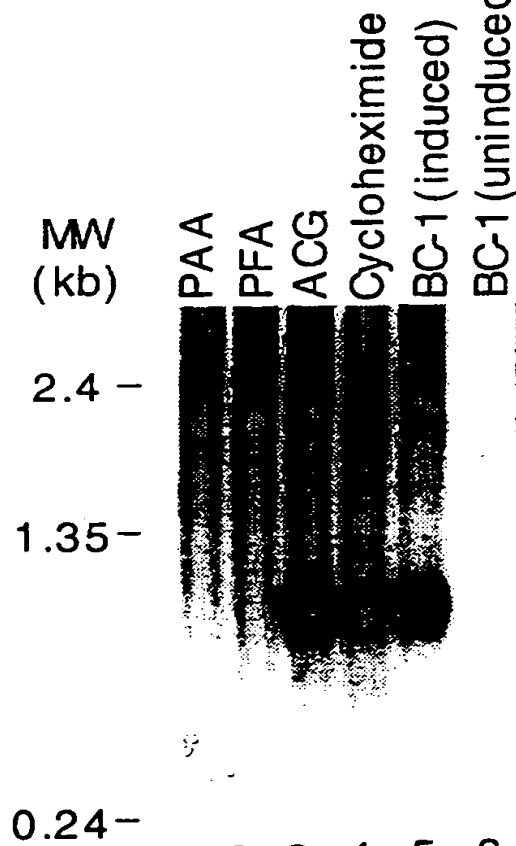
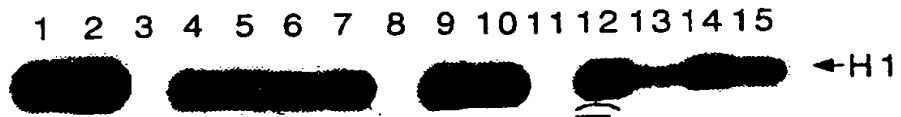
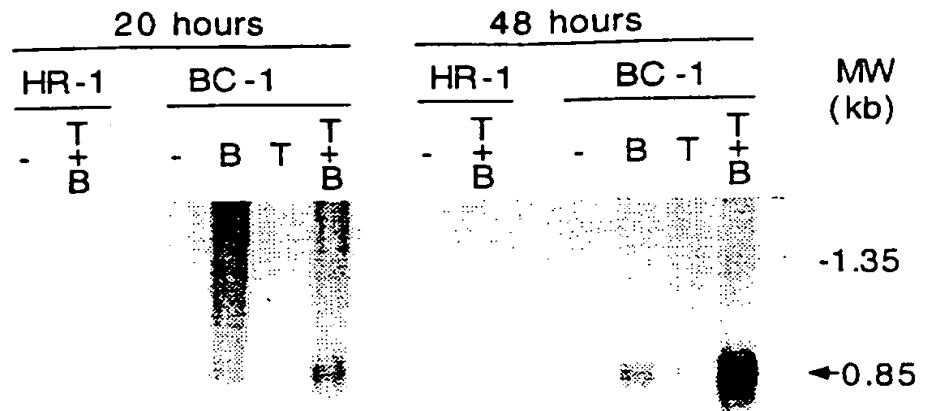
-120 attcgagacctacatcaaactgtcagttttgtagtgccttgccttgggacgcccgactgc  
-60 gaggctgcctattaagcaccgtgacgtgcgcgaggactgcttcagctcacctcacagctg  
1 CTGGAGTTCGTGTTGCGGGAAGTGTTCCTCCTGAGGCTATTTGCCCCGCTGTGTGGAAGA  
61 TGTCCAACTTTAAGGTGAGAGACCCCGTGATCCAGGAGCGACTGGATCATGACTACGCTC  
S N F K V R D P V I Q E R L D H D Y A H M  
121 ACCATCCCCTGGTGGCTCGCATGAATACCCTGGATCAGGGAATATGTCGCAGGCCGAAT  
H P L V A R M N T L D Q G N M S Q A E Y  
181 ACCTCGTGCAAAGAGACACTATCTCGTGTTCCTTAATTGCCCATCATTATTATGAGGCC  
L V Q K R H Y L V F L I A H H Y Y E A Y  
241 ATCTGAGGAGGATGGGTGGAATTCAAAGGCGGGATCATCTGCAGACCCCTTAGGGATCAGA  
L R R M G G I Q R R D H L Q T L R D Q K  
301 AACCGCGGAGCGCGCTGACCGAGTTCCGCGGGCGTCCGCTTACGACGCGGGGACGTTCA  
P R E R A D R V S A A S A Y D A G T F T  
361 CCGTGCCTTCGAGGCCAGGCCCTGCATCCGGCACCACGCCCCGGGGCCAGGACTCACTGG  
P S R P G P A S G T T P G G Q D S L V G  
421 GGGTCTCGGGAAGCAGTATAACCACCCTGTCCCTCTGGTCCCCATTTCATTGTGCGCCGCGT  
V S G S S I T T L S S G P H S L S P A S  
481 CAGACATTCTCACAACCCTCTCATCCACGACGGAAACGGCCGCCCCCGGGTGGCCGACG  
D I L T T L S S T T E T A A P A V A D A  
541 CGAGGAAACCCCTCTGGCAAAAAGAAATAGGCAACGATTGGACAACCGTGGAGTCACA  
R K P P S G K K K \*  
601 AGTACCCTTCTTTATTCTGTCAACCGTCTCCGCCAGGGACGCCGATAGGGCGGCCACTT  
661 TTTGTTTGATGCGTCTAATAAACTAATCAGTGTATCAAAAAAAAAAAAAAAAAAAAA

Figure 12

Hv8orf65	MHR	L	R	V	T	D	P	V	V	Q	G	L	E	E	S	D	S	T	H	E	L	V	K	R	L	E	I	L	P	Q	N	N	M	T	P	E	E	40		
Kshvsvca	MSN	F	V	R	D	P	V	I	Q	R	L	N	D	Y	A	H	H	P	L	V	A	R	M	N	T	L	D	Q	Q	C	N	M	S	Q	A	E	40			
Ebvbfif3	MAR	R	P	K	P	T	L	O	G	R	L	E	A	D	F	D	S	P	L	P	P	F	Q	E	L	N	Q	N	N	P	N	D	V	39						
Hv8orf65	Y	T	L	M	K	R	N	Y	L	V	F	L	I	A	Q	Y	N	Q	Y	I	E	T	H	G	I	K	R	K	H	I	E	G	L	77						
Kshvsvca	Y	L	V	Q	K	R	H	Y	L	V	F	L	I	A	H	Y	Y	E	A	Y	L	R	R	M	G	I	Q	R	D	H	L	Q	T	L	R	D	Q	80		
Ebvbfif3	R	E	A	Q	R	S	Y	L	V	F	L	T	S	Q	F	C	Y	E	Y	V	Q	R	T	F	G	P	R	R	Q	M	A	I	D	K	R	Q	R	79		
Hv8orf65	K	A	N	L	K	S	A	L	S	A	D	S	A	S	L	S	G	L	L	T	V	P	S	R	P	G	P	A	S	L	T	T	P	S	105					
Kshvsvca	K	P	R	E	R	A	D	R	V	S	A	A	S	A	Y	D	A	G	T	F	Q	Q	A	Q	A	A	S	A	S	A	S	G	T	T	P	G	Q	D	S	119
Ebvbfif3	A	S	V	A	G	A	G	A	H	A	H	L	S	S	S	A	T	P	V	Q	S	T	S	S	T	S	T	G	A	L	S	S	A	P	S	119				
Hv8orf65	L	G	V	S	G	S	S	I	T	T	L	S	S	T	P	T	S	L	T	S	M	P	G	L	S	I	S	G	P	S	T	T	D	T	130					
Kshvsvca	T	A	V	A	Q	S	A	T	P	S	L	S	S	G	P	H	S	L	S	P	A	S	D	I	L	T	T	L	S	S	T	T	E	T	A	P	A	V	A	159
Ebvbfif3	T	A	V	A	Q	S	A	T	P	S	L	S	S	S	S	I	S	S	L	R	A	A	T	S	G	A	T	A	A	A	A	V	D	T	G	S	G	159		
Hv8orf65	D	S	K	K	K	P	K	A	K	139																														
Kshvsvca	D	A	R	K	P	P	S	G	K	K	170																													
Ebvbfif3	G	G	Q	P	H	D	T	A	P	R	G	A	R	K	K	Q	176																							

Figure 13





1 2 3 4 5 6  
**Figure 14**  
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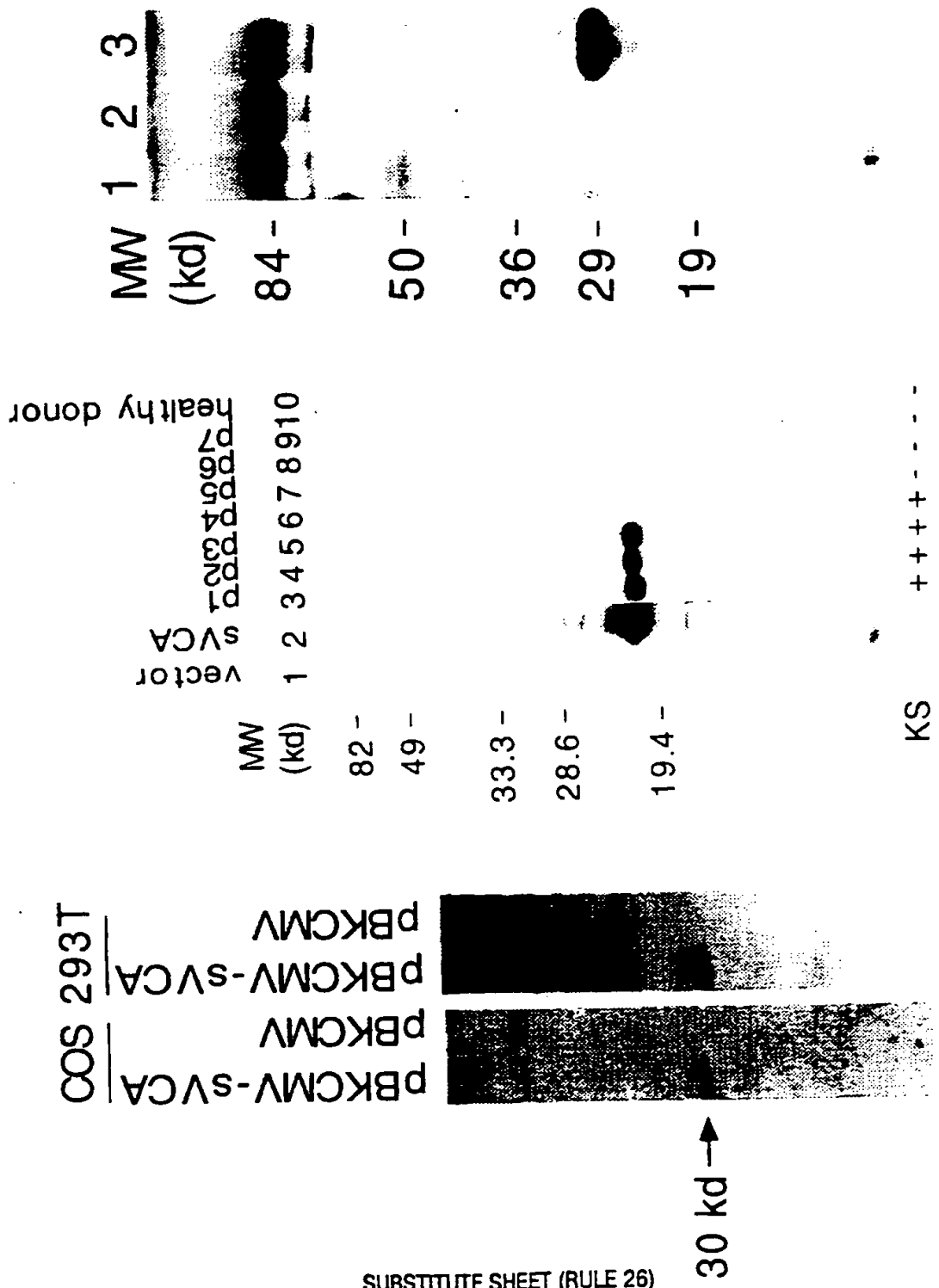


Figure 15

1 2 3 4

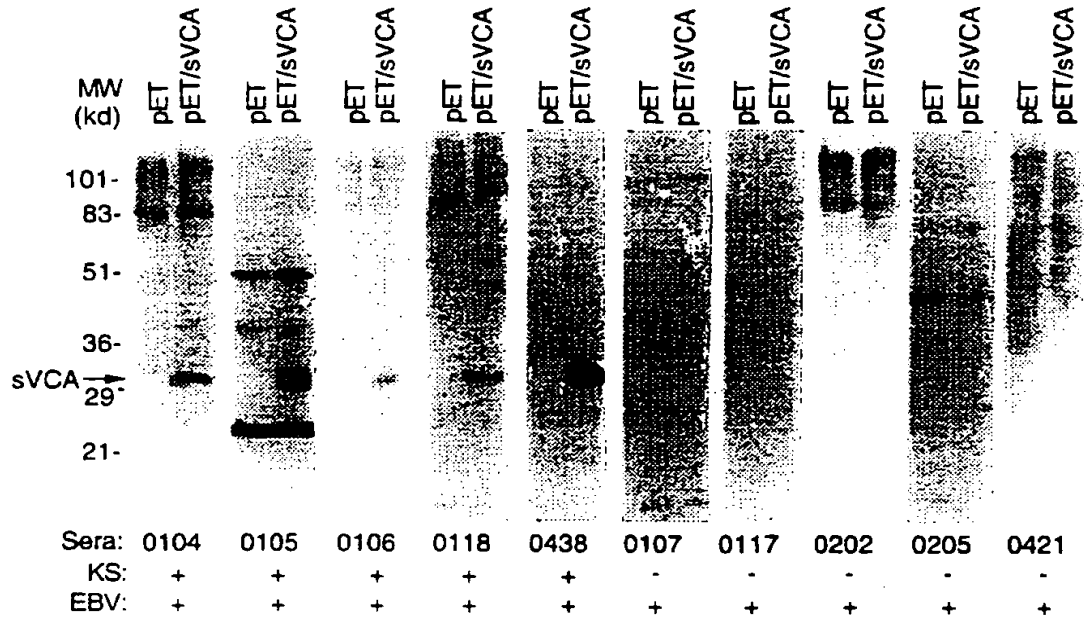


Figure 16

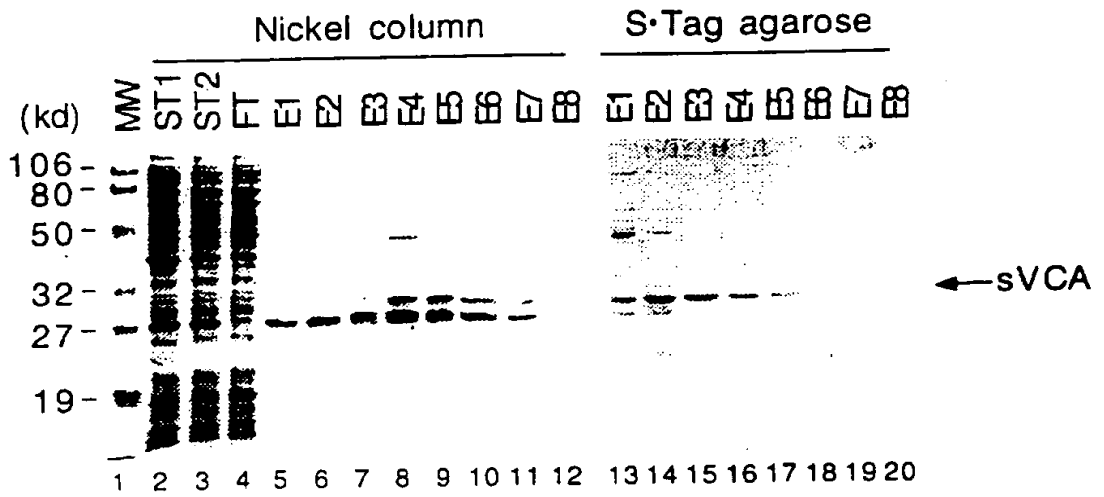
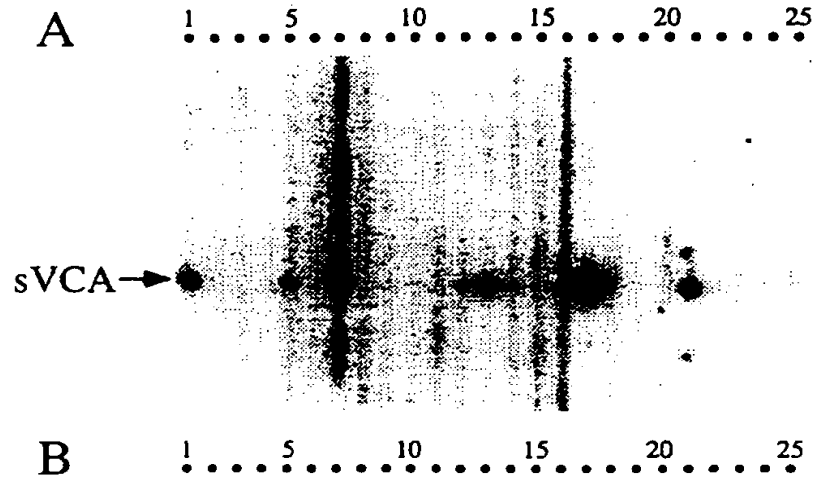


Figure 17



**Figure 18**

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